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15206

A Simple Method for the Concentration of Rh Agglutinins.

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There are two sources of human anti-Rh serum. Following multiple transfusions of Rh-positive blood certain Rh-negative individuals develop Rh antibodies.[1] The more common source of human Rh antibodies, however, is found in women who have given birth to erythroblastotic children.[2] Rh agglutinins if present are frequently of low titer. The procedure for fractionated precipitation of sera containing antibodies has been developed especially in Dr. Cohn's laboratory at Harvard, and is successfully applied in the case of anti-Rh serum (Diamond). However, this method requires special equipment and training and is not available to everybody. It seemed to be desirable to find means and ways to concentrate Rh agglutinins by a simple method.

Anti-Rh sera were subjected to dialysis in the following manner: The native serum is placed in a cellophane tubing bag under tension, air having been eliminated as much as possible. The bag is then suspended in a battery jar containing a large volume of freshly distilled water. The serum is dialyzed 24 to 30 hours in the icebox at 4°C, the distilled water being changed several times during the procedure. During dialysis at this temperature a precipitate forms. The serum with its precipitate is removed from the cellophane bag and the precipitate is separated from the supernatant fluid by centrifugation. In the experiments to be described, the sediment (precipitate) is dissolved in saline solution and referred to as the globulin fraction. The globulin fraction obtained from 10 cc of each serum is dissolved in 1 cc of saline solution (0.9% NaCl) by vigorous stirring, then kept for 2-3 hours in the icebox. Insoluble particles are removed by centrifugation. The dissolved globulin is preserved in the frozen state at –20°C or

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[1] Wiener, A. S., and Peters, H. R., *Ann. Int. Med.*, 1940, **13**, 2306.

[2] Levine, Philip, *Am. J. Obs. and Gyn.*, 1945, **49**, 810.

TABLE I.
Agglutination of Rh Positive Group O Cells by Serum (Dem).

Serum (Dem)	a Native	b Supernatant	c Globulin
1. Undiluted	++++	+++	++++
2. 1:2	+++	++	++++
3. 1:4	+++	+	++++
4. 1:8	+++	+	++++
5. 1:16	++	±	++++
6. 1:32	++	—	++++
7. 1:64	+	—	+++
8. 1:128	±	—	+++
9. 1:256	—	—	++
10. 1:512	—	—	++
11. 1:1024	—	—	±
12. Saline	—	—	—

— = No agglutination.
± = Faint agglutination.
+ = Slight agglutination.
++ = Marked agglutination.
+++ = Strong agglutination.
++++ = Very strong agglutination.

lyophilized. The supernatant fluid to which a sufficient amount of 10% saline solution is added to bring the final salt concentration to 0.9% is referred to in this paper as the "supernatant." In former years the described procedure served as a rough method to separate the globulin and the albumin fractions of serum. It is now generally agreed that the albumin fraction obtained by dialysis is by no means pure but still contains some globulin.

The analysis of several anti-Rh sera is reported in this paper. One serum (Kru) was obtained from an Rh-negative patient who had received multiple transfusions and who had died after a long chronic illness. A large amount of blood containing an Rh agglutinin of relatively weak potency was collected after the patient's death. The second serum (Dem) and the third serum (Heng) were obtained from Rh-negative women who had given birth to erythroblastotic children; so was a fourth serum which contained "blocking antibodies" only.[5]

The first experiment was carried out in the following way: Decreasing amounts of serum (Dem) and its fractions respectively (volume 0.1 cc) were mixed with 0.1 cc of a 2% suspension of Rh-positive Group O cells. After standing at room temperature for one hour the tubes were centrifuged at medium speed for 1 minute and read for agglutination.

The experiment shows that the globulin fraction, dissolved in one-tenth of the original volume of the serum, contains a potent Rh agglutinin. Characteristic of the agglutination of Rh-positive cells by the globulin fraction is not only an increase in end titer but also an increase in the relative strength or quality of the agglutination showing a 4+ agglutination up to a dilution of 1:32. In contrast, the supernatant fluid is poor in Rh agglutinins when compared with the native untreated serum. From this experiment the conclusion can be drawn that the Rh agglutins are found in the "globulin" fraction as obtained by simple dialysis and only a minor portion remains in the supernatant fluid.

The discovery of the "incomplete antibody" (Race[3,4]) or the "blocking antibody" (Wiener[5]) in the serum of patients expected to contain Rh antibodies has proved to be of great importance. This antibody uniting with Rh-positive cells, thus sensitizing them, will prevent agglutination of Rh-positive cells by the Rh agglutinin. Diamond's and Abelson's[6] investigations have shown that "blocking antibodies" when mixed with whole blood will lead to agglutination of Rh-positive cells on the slide as well as in the test tube. As demonstrated by Diamond and his co-workers[7,8] and Wiener,[9] agglutination of Rh-positive cells by "blocking antibodies" can be easily obtained if undiluted serum or 20% albumin replaces saline solution as a diluent.

The following experiment as recorded in Table II deals with serum (Kru) and its fractions derived by dialysis. The experiment itself was carried out in two parts. In the first part, all dilutions were made in saline solution, and for the second part all dilutions

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[3] Race, R. R., *Nature*, 1944, **153**, 771.

[4] Race, R. R., and Taylor, G. L., *Brit. Med. J.*, 1944, **2**, 756.

[5] Wiener, A. S., Proc. Soc. Exp. Biol. and Med., 1944, **56**, 173.

[6] Diamond, L. K., and Abelson, N. M., *J. Lab. and Clin. Med.*, 1945, **30**, 204.

[7] Diamond, L. K., and Abelson, N. M., *J. Lab. and Clin. Med.*, 1945, **30**, 668.

[8] Diamond, L. K., and Denton, R. L., *J. Lab. and Clin. Med.*, 1945, **30**, 821.

[9] Wiener, A. S., *J. Lab. and Clin. Med.*, 1945, **30**, 662.

TABLE II.
Agglutination of Rh_1 Group O Cells by Serum (KRU).

	I. All dilutions in saline			II. All dilutions in serum		
Serum (KRU)	a Native	b Supernatant	c Globulin	a Native	b Supernatant	c Globulin
1. Undiluted	++++	—	++++	++++	++	++++
2. 1:2	+++	—	++++	+++	++	++++
3. 1:4	+++	—	++++	+++	++	++++
4. 1:8	++	—	++++	++	+	++++
5. 1:16	+	—	++++	+	±	++++
6. 1:32	±	—	++++	±	±	+++
7. 1:64	—	—	+++	—	—	+++
8. 1:128	—	—	++	—	—	++
9. 1:256	—	—	+	—	—	+
10. 1:512	—	—	—	—	—	±
11. 1:1024	—	—	—	—	—	±
12. Saline	—	—	—	—	—	—

(cells, serum and its fractions) were made in undiluted serum.

Decreasing amounts of serum (Kru) and its fractions (volume 0.1 cc) were mixed with 0.1 cc of Rh_1 cells belonging to Group O. After standing for one hour at room temperature the tubes were centrifuged and read.

Experiment II again demonstrates the fact that Rh agglutinins appear in the globulin fraction. In this case the "supernatant" appears to be completely free of agglutinins. However, when diluted in serum the "supernatant" reveals the presence of antibodies of the type referred to as "blocking" or "incomplete antibodies." It should be pointed out that the untreated serum when diluted in serum does not reveal a higher antibody titer than when diluted in saline solution. One might therefore come to the conclusion that serum (Kru) is free of "blocking antibodies," but actually the presence of such antibodies can be demonstrated by splitting the serum into fractions by dialysis and using serum as a diluent instead of saline. The relative effectiveness of the globulin fraction in respect to the agglutination of Rh-positive cells in a saline medium can therefore be explained not only by concentration of the Rh agglutinins, but also by the fact that the accompanying "blocking antibodies" have not been concentrated to the same extent. The same results as those reported in Table II were observed when Rh_2 and Rh_o cells were used instead of Rh_1 cells.

The presence of "blocking antibodies" in serum (Kru) manifests itself easily if a different order of experiment is used as shown in Experiment III.

Decreasing amounts of (a) serum obtained from an Rh-negative mother who had an erythroblastotic child and whose serum contained "blocking antibodies" only, (b) a normal serum (as a control), (c) the supernatant fraction of serum (Kru) (volume 0.15 cc) were mixed with 0.05 cc of a 6% suspension of Rh_1 cells belonging to Group O. After incubation for 30 minutes in the water bath at 37°C, 0.05 cc of undiluted anti-Rh serum (Diamond B-2X) were added. The tubes after being shaken well were kept for an additional hour at 37°C, and centrifuged.

The blocking effect of the supernatant (Kru) which equals in strength the blocking property of an untreated serum of an erythroblastotic woman known to contain "blocking antibodies" is evident from this experiment. The question arose whether the "incomplete" or "blocking antibody" was to be found also in the globulin fraction, or whether this antibody would remain in the supernatant following dialysis. For that reason serum (Heng) containing "blocking antibodies" exclusively was selected and subjected to dialysis, using the technic as described above. The untreated serum as well as the two serum fractions obtained by dialysis were then examined in the following way:

Decreasing amounts of whole serum (Heng) and its serum fractions respectively (volume 0.1 cc) were mixed with 0.1 cc of a 2%

TABLE III.
Inhibitory Effect of "Blocking Antibodies" on Agglutination of Rh_1 Cells by Rh Agglutinins.

Serum containing blocking antibodies	a Blocking serum	b Normal serum control	c Serum (KRU) supernatant
1. Undiluted	—	+++	—
2. 1:3	—	+++	—
3. 1:9	+	+++	±
4. 1:27	++	+++	+
5. 1:81	+++	+++	++
6. 0	+++	+++	+++

suspension of Rh_1 cells belonging to Group O. The experiment was carried out in two parts: Serum dilutions and blood cell suspensions were made (I) with saline solution and (II) with undiluted normal human serum. Tubes were kept for one hour in a water bath at 37°C, and then centrifuged for one minute at a medium speed. The resulting agglutination is shown in Table IV.

The native serum (Heng) as well as the serum fractions obtained by dialysis do not give any visible agglutination with Rh_1 cells Group O when diluted in saline solution. However, if undiluted normal serum is used as a diluent, agglutination of Rh-positive cells occurs in rather high dilutions. The fraction referred to as "supernatant" shows definite agglutination indicating that the "blocking antibody" is present almost to the same extent as in the untreated serum. The globulin fraction also contains some "blocking antibody." Considering the fact, however, that we are dealing here with an approximately 10 times concentrated fraction it becomes obvious that a large portion of the "blocking antibody" has remained in solution (supernatant) following dialysis.

The serum fractions obtained by dialysis were also examined for the presence of isoagglutinins anti-A and anti-B. Somewhat irregular results were obtained. The impression was that dialysis would divide the isoagglutinins more or less equally between the globulin fraction and the supernatant, though in many instances the supernatant fluid contained more isoagglutinins anti-A and anti-B than the globulin fraction.

Conclusions. The dialysis of serum containing Rh antibodies permits the division of such a serum into two fractions, the precipitate, containing mostly globulins, and the supernatant, containing the albumin fraction and a certain amount of the globulin fraction. The sediment, when dissolved in physiological saline solution using one-tenth of the original volume of the serum prior to treatment, is shown to contain the major portion of the Rh agglutinins. A rather potent Rh testing serum can be obtained by this procedure, where in many instances anti-Rh sera contain agglutinins of such weak potency that the native

TABLE IV.
Titration of Rh "Blocking Antibodies" in the Different Fractions of Serum (Heng) Against Rh_1 Cells.

	a Native		b Supernatant		c Globulin	
	I. Diluted with saline	II. Diluted with serum	I. Diluted with saline	II. Diluted with serum	I. Diluted with saline	II. Diluted with serum
1. Undiluted	—	+	—	+	—	++
2. 1:2	—	++	—	++	—	+
3. 1:4	—	+++	—	++	—	+
4. 1:8	—	++	—	+++	—	+
5. 1:16	—	++	—	+++	—	++
6. 1:32	—	++	—	++	—	++
7. 1:64	—	++	—	++	—	++
8. 1:128	—	+	—	+	—	++
9. 1:256	—	±	—	±	—	++
10. 1:512	—	—	—	±	—	+
11. 1:1024	—	—	—	±	—	+
12. Saline	—	—	—	—	—	—

serum would not be strong enough to serve as a diagnostic test serum. The method is especially recommended for this purpose. The "incomplete" or "blocking antibody" has been found to be associated to a large extent with the supernatant, indicating that at least a partial separation of the Rh agglutinin from the "blocking antibody" has been accomplished by dialysis. The effectiveness of the resulting Rh agglutination by the globulin fraction is therefore not only attributed to simple concentration of the Rh agglutinins, but also to the partial removal of the Rh "blocking antibody." The isoagglutinins anti-A and anti-B are not concentrated to the same extent as the Rh agglutinins which constitutes an additional advantage of the globulin preparation over the untreated serum.

15207

Results of Inoculating Okinawan Horses with the Virus of Japanese B Encephalitis.

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It has been reported previously[1] that a considerable percentage of Okinawan horses, from which samples of serum were obtained during an epidemic of Japanese B encephalitis, possessed antibodies against the virus responsible for the epidemic. This observation suggests that horses may occupy the role of intermediary hosts for the virus. The importance of this role in the epidemiology of the disease in human beings depends to a large extent upon whether the virus is capable of circulating in the blood stream of infected horses, from which source it might be transmitted to man by mosquitoes. In order to obtain information concerning the matter, four Okinawan horses were inoculated with the virus of Japanese B encephalitis on 16 September, 1945. The present report is concerned with the results of the experiment.

Materials and Methods. Through the cooperation of the Commander of Naval Construction Troops, a screened, wooden-frame stable was built on the outskirts of the village of Ishikawa. This building contained four stalls, a separate room for laboratory studies, and a concrete floor with drainage outlets leading to a nearby pit. Throughout the course of the experiment, the interior was thoroughly sprayed each day with DDT.

Four horses were selected from the corral at Ishikawa. Like virtually all other horses on Okinawa, these were malnourished, undersized animals whose general physical condition was obviously poor. Three of the horses were approximately one year old; the fourth was a Mongolian pony approximately 12 years old. Each horse was inoculated intravenously with 2 cc of a 10% suspension of mouse-brain tissue, infected with a strain of Japanese B encephalitis virus isolated during this epidemic from a fatal case of encephalitis.[2] Specimens of blood were obtained from each horse before the injection of virus and at intervals of 16 hours, 3, 6, 9, and 12 days after inoculation. Neutralization tests were performed with the pre-inoculation sera in order to determine the status of immunity in each animal at the time of injection. The specimens of blood taken after inoculation were injected intracerebrally and intraperitoneally into mice, in order to determine the presence or absence of virus. In the instances where a virus was recovered

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* The ideas set forth herein are those of the authors and are not necessarily those of the Bureau of Medicine and Surgery of the Navy Department.

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[2] Hodes, H. L., Thomas, L., and Peck, J. L., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 220.

TABLE I.
Results of Neutralization Tests and Mouse Inoculations with the Blood of Horses Inoculated with Japanese B Encephalitis Virus.

Horse No.	Neutralizing titer*	Virus in blood				
		16 hr	3 days	6 days	9 days	12 days
1	0	0	+	+	0	—
2	1000	0	0	0	0	0
3	1000	0	0	0	0	0
4	1000	0	0	0	0	0

* Titer is expressed as number of 50% mortality doses of virus against which each serum protected mice.

from the blood stream, it was subsequently identified as that of Japanese B encephalitis by means of neutralization tests in mice.

Results. The results of neutralization tests with sera obtained prior to inoculation, and the results of the injection of mice with blood taken following inoculation, are shown in Table I. It will be seen that Horse No. 1, a yearling, did not possess demonstrable neutralizing antibodies in the serum obtained before inoculation. Sixteen hours after injection, the virus had apparently disappeared from the circulating blood, as shown by the negative results obtained by the injection into mice of blood removed at this time. However, 3 days following inoculation, the virus had reappeared in the blood, since each of 3 mice, which were injected with blood removed on the third day, developed typical symptoms of encephalitis and died 6 days after injection. The virus in the brains of these mice was identified as that of Japanese B encephalitis by means of neutralization tests with known hyperimmune Japanese B antiserum. On the 6th day after the inoculation of Horse No. 1, virus was again detected in the blood of the animal and subsequently identified as the virus of Japanese B encephalitis. On the 9th day, virus was not demonstrated in the blood of Horse No. 1. It is noteworthy that, on this day, its serum was proved to contain sufficient antibody to neutralize 100 50%-mortality doses of virus in mice. It is possible that the failure to demonstrate virus in the blood on this day may have been due to a masking of small amounts of virus by antibody.

Horse No. 1 became ill on the sixth day, failing to eat and showing generalized weakness. The weakness increased during the next four days, and on the 10th day the animal was moribund. At this time it was sacrificed, and specimens of tissues were obtained for inoculation of mice as well as for histopathological examination. Virus could not be recovered from the brain, spinal cord, or spleen of this horse, and microscopic examination of the brain showed no evidence of encephalitis. The cause of the death of this horse is not known; it is possible that it may have been due in part to prolonged malnutrition.

On the day of the experimental inoculation, Horses No. 2, No. 3, and No. 4, as is shown in Table I, each possessed antibody in sufficient titer to neutralize 1000 50%-mortality doses of virus in mice. As might be expected, virus was not recovered from the blood of any of these animals at any time up to the 12th day after inoculation. These horses developed no symptoms of infection and remained entirely well for a period of 20 days after inoculation.

Summary. It has been demonstrated that an Okinawan horse was susceptible to infection by the virus of Japanese B encephalitis and that the virus was present in the circulating blood 3 and 6 days after inoculation. It could not be demonstrated 16 hours after inoculation, nor after 9 days, at which time a small amount of neutralizing antibody had appeared. In 3 other horses which possessed high titers of neutralizing antibodies prior to the injection, virus could not be demonstrated in the blood stream following inoculation. These results, when considered in conjunction with the previously demonstrated presence of high titers of antibodies in the sera of many Okinawan horses,[1] constitute further evidence that this species of animal may play a role in the epidemiology of Japanese B encephalitis.

15208

Relationship of Carotid and Aortic Mechanisms to Digitalis Emesis.*

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The site of the emetic action of digitalis and related compounds has been a subject of considerable research and controversy over a period of more than 30 years. The initial conclusions of Hatcher and Eggleston,[1,2,3] that the effect was of central rather than peripheral origin, were not borne out by later studies.[4,5] Hatcher and Weiss[4,6,7] and Hatcher and French[8] suggested that the emetic response to digitalis was of a reflex nature initiated by afferent impulses arising in the heart. This was later questioned by Dresbach and Waddell,[9,10,11] and by Haney and Lindgren.[12] The latter authors conclude that the mechanism must lie either in a direct action on the medullary center, as originally suggested, or "on other structures from which impulses pass to the center via nerves other than those eliminated in the experiments described." The liver, as a possible source of such impulses, has been eliminated by Dresbach.[13] Hanzlik and Wood concluded that, in spite of the fact that digitalis emesis occurred in their dehepatized pigeons, the liver is still the primary source of the reflex.[14] The gastro-intestinal tract was eliminated by Hatcher and Eggleston.[1]

It is well known the muscles of respiration are definitely concerned with vomiting[15] and that excitation of the mechanisms located in the carotid and aortic bodies and indirectly through the carotid sinus and arch of the aorta may, under proper circumstances, result in respiratory stimulation. It has also been noted[9] that in cases where emesis does not result from digitalis administration *rapid respiration* frequently occurs. The present study was undertaken in order to determine whether or not the surgical elimination of the carotid and aortic mechanisms or their nervous pathways might result in abolishing the emetic response. An abstract of the work herein reported appeared recently.[16]

Method. A single intravenous dose of digitoxin, which would produce emesis within 30 minutes, was established. Three dogs were given 0.09 mg/kg digitoxin. At the end of one hour, none of the animals having vomited, each dog was given one-half of its original dosage. Emesis occurred in all animals within 30 minutes. Three dogs were, therefore, given the approximate sum of these doses (0.15 mg/kg). Emesis occurred in all animals within 30 minutes. Hence, in all subsequent experiments this was the vomiting dose employed.

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* The author is indebted to Dr. H. B. Haag for his valuable counsel during the course of this work and to Dr. E. L. Smith who assisted with the surgical procedures.

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TABLE I.
Effect of Removal of Carotid Bodies and Sinuses and Denervation of the Aortic Regulators on the Emetic Response to 0.15 mg/kg Digitoxin on Dogs.

Dog No.	Date	Treatment	Emesis after digitoxin in
1 ♂	4-12		22 minutes
	20	Carotids removed	
	26		24 "
2 ♀	5-24		18 "
	29	Carotids removed	
	6-3		6 "
	9	Right vagus cut	
	12		17 "
3 ♀	5-29		20 "
	6-5	Carotids removed	
	12		16 "
	19	Right vagus cut	
	23		15 "
4 ♀	12-18		16 "
	21	Carotids removed, left vagus cut	
	27		25 "
	1-10	Right vagus cut	
	11		25 "
5 ♂	12-27		12 "
	28	Carotids removed, left vagus cut	
	1-3		12 "
6 ♀	12-29		6 "
	30	Carotids removed, left vagus cut	
	1-13		22 "
7 ♂	12-30		15 "
	31	Carotids removed, left vagus cut	
	1-6		27 "
	11	Right vagus cut	
	13		16 "

Seven dogs (4 females, 3 males) were each checked for the normal emetic response to 0.15 mg/kg digitoxin. After allowing a minimum of 24 hours for recovery, the animals underwent the following operations in 2 or more steps as indicated by Table I: (a) removal of the carotid bodies and sinuses by extirpation of segments of the carotid arteries from points just caudal to the origin of the superior thyroid artery to points slightly cephalic to the origin of the lingual artery, (b) sectioning of one or both vagal trunks (in the neck region) thus interrupting the nervous pathways from the aorta.[17] All operations were performed under ether anesthesia.

Each animal was given a minimum of 24 hours to recover from operation and at least 7 days to recover from the previous dose of digitoxin. The dog was then given 0.15 mg/kg digitoxin and watched for 30 minutes for emesis. This was repeated after each phase of the operation.

The results are shown in Table I. In every case vomiting was as prompt and typical after operation as before. Six of these dogs survived sectioning of one vagus and 2 survived sectioning of both vagi without change in their vomiting response to digitoxin. This is contrary to the experience of Hanzlik and Wood[14] who found that double vagotomy abolished digitalis emesis in pigeons. It is in agreement, however, with the results of Haney and Lindgren[13] on dogs.

Conclusion. Vomiting initiated by digitoxin is not abolished by removal of the carotid bodies and sinuses and by denervation of the aortic mechanisms. Assuming the mechanism to be reflex in nature, the site of origin of the impulses causing digitalis vomiting is still undetermined. The possibility remains that afferent impulses may be set up in a structure or structures not yet eliminated. It is perhaps more probable that impulses may arise from any one of several structures all of which would have to be denervated in the same animal to abolish the emetic response to digitalis.

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15209

The Electron Micrography of Plant Virus-Antibody Mixtures.

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Electron microscopy is a promising new way of studying the interaction between an antigen and its specific antibody. Such a study is of practical importance because the minute amounts of required material make possible the development of unusually sensitive tests for antigen and antibody. From a more fundamental standpoint the ability of the microscope to record particles comparable in size to antibody molecules may provide a way of checking directly the several hypotheses that have been developed to explain the nature and specificity of the immune reaction.

Very few papers dealing with the electron microscopy of antigen-antibody mixtures have thus far been published. In two[1,2] the antigens are bacteria; another[3] describes the apparent swelling of tobacco mosaic virus particles when mixed with antibody. The published pictures of sensitized typhoid and paratyphoid bacilli give clear evidence of the adsorption of antibody onto both the cell walls and the flagella. In the tobacco mosaic-serum photographs isolated fibers show the same kind of thickening as these bacterial flagella. It was suggested[3] this broadening of the tobacco mosaic particles, from a normal 150A to *ca.* 600A, was due to each being coated with a single layer of antibody molecules arranged with their long axes at right angles to the axis of the virus particle.

The visibility of viruses[4] and other objects of macromolecular dimensions[5] is improved by the oblique evaporation upon the preparation of a very thin metallic film.[6] We are using this "shadow" technic to see what new information it will give concerning the reaction between certain plant viruses and sera prepared against them. Preliminary photographs are reproduced in this note.

Antigens used were tomato bushy stunt and southern bean mosaic viruses purified by means of a preliminary clarification with $(NH_4)_2SO_4$ followed by fractionation in a high speed centrifuge. Antisera for the bushy stunt virus and for the bean virus[7] were prepared by intraperitoneal injection of rabbits with puri-

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[4] Williams, R. C., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 265; *Science*, 1945, **101**, 594; Price, W. C., Williams, R. C., and Wyckoff, R. W. G., *Science*, 1945, **102**, 277.

[5] Williams, R. C., and Wyckoff, R. W. G., *Nature*, 1945, **156**, 68.

[6] Williams, R. C., and Wyckoff, R. W. G., *J. Applied Physics*, 1944, **15**, 712; 1946, **17**, No. 1.

[7] Price, W. C., and Black, L. M., *Phytopathology*, in press.

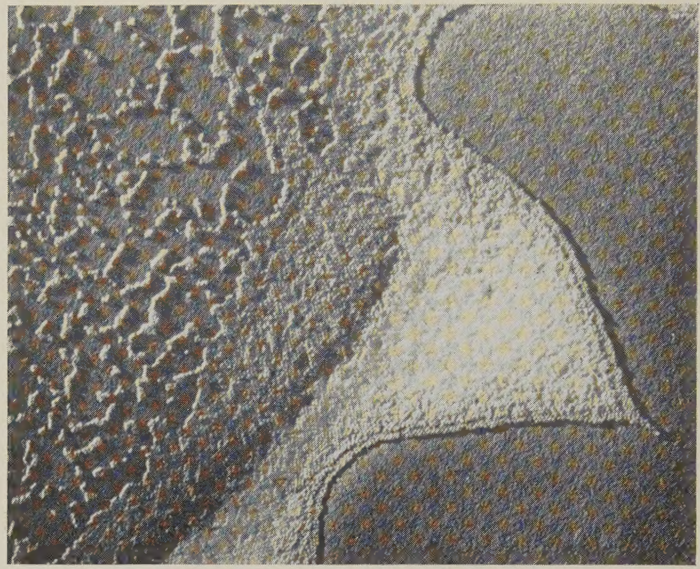

FIG. 1.

An electron micrograph of a field in a preparation of purified southern bean mosaic virus. At the top of the picture the bean virus particles, singly and in groups, are widely separated from one another on the substrate. In the middle they are more closely packed into a nearly continuous layer. At the bottom these layers of particles are stacked on top of one another to give the beginnings of a crystalline array. Magnification 16,800 ×.

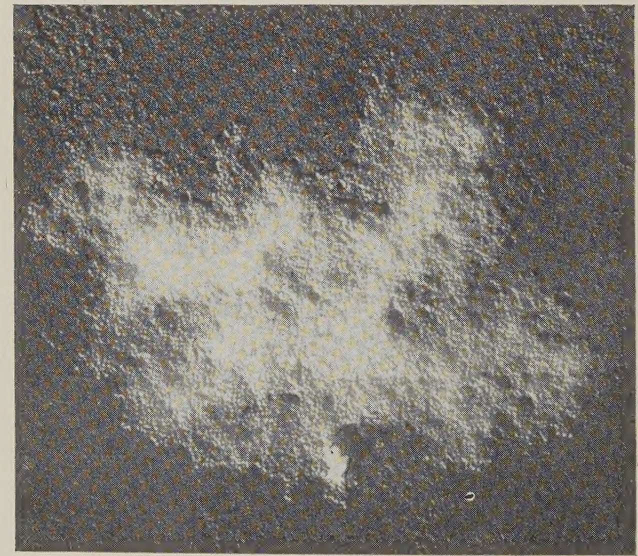

FIG. 2.

An electron micrograph of a field in a preparation consisting of a mixture of purified southern bean mosaic virus with its antiserum. The individual particles in the floc do not have the same degree of regularity in arrangement that is evident in Fig. 1. Magnification 16,800 ×.

fied virus suspensions. The anti-bushy stunt serum was originally made for another set of experiments for which it was important that the serum be freed from traces of tobacco mosaic antiserum that might be present. To insure this it has been absorbed with purified tobacco mosaic virus, the excess of which provides the rod-forms seen in Fig. 3.

Preparations for electron microscopy were made in the following way. Purified virus containing *ca.* one milligram per cc was mixed with an equal volume of undiluted serum and the mixture immediately diluted with from 10 to 100 volumes of distilled water. This diluted serum-virus mixture was examined immediately and after varying intervals up to 24 hours. Time seemed to have little effect on the character of the electron photograph obtained. The concentrated serum-virus mixture soon became turbid with the eventual precipitation of a part of its contents but the more dilute mixtures remained clear through-

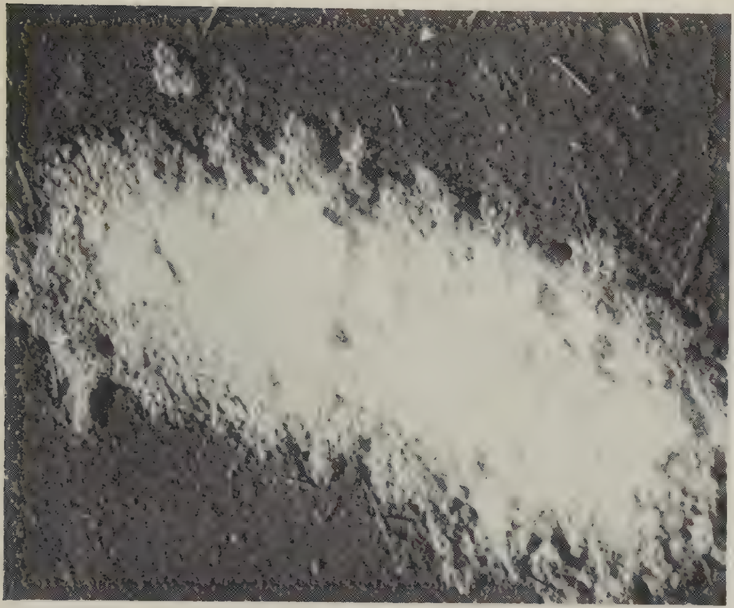

FIG. 3.
An electron micrograph of a similar preparation consisting of a mixture of purified bushy stunt virus with antiserum. Magnification 20,500 ×.

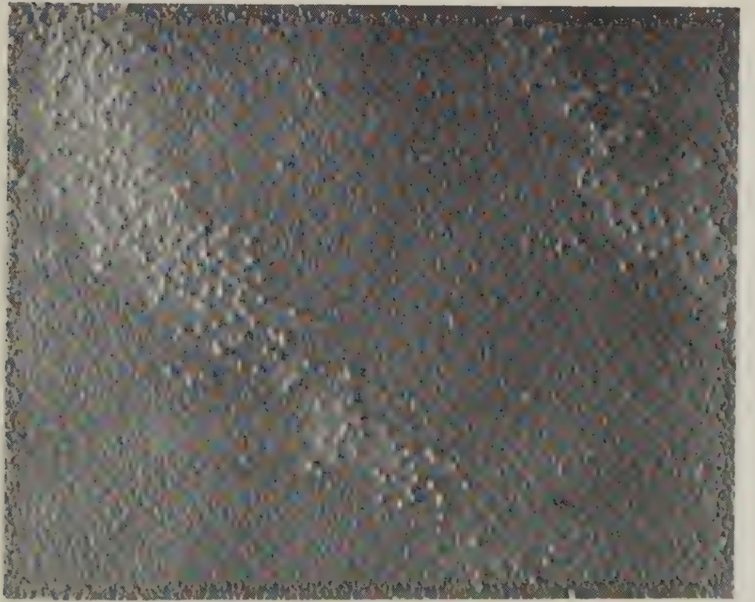

FIG. 4.
An electron micrograph of a field of a preparation consisting of a mixture of bushy stunt virus with normal rabbit serum. Magnification 20,500 ×.

out the period of observation. Micro-drops of the diluted mixtures were placed on the usual collodion-covered screens, left several minutes and then withdrawn as completely as possible. After being allowed to dry in the air the screens containing the less dilute mixtures were washed several times by the addition and subsequent withdrawal of droplets of distilled water. Final preparations were shadowed as usual in the vacuum chamber of a metal-evaporating unit.§ Gold in an average thickness of *ca.* 8A was laid down, the angle of evaporation being such that an object on the preparation would cast a shadow five times its height.

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§ We wish to express our gratitude to Perry C. Smith and R. G. Picard of the Radio Corporation of America, Camden, N.J., for the use of evaporating equipment.

Two sorts of preparations were made and examined as controls for the foregoing virus-antibody mixtures. One consisted of mixtures containing normal in place of immune serum, the other was of serum without admixed virus. These were shadowed in the usual fashion before examination.

Both viruses have elementary bodies that are essentially spherical; in pure suspension and at sufficient concentrations they show a pronounced tendency to associate in regular crystal-like arrays.[8] When mixed with specific antiserum, however, these particles appear aggregated without the same regularity into microclumps. The character of this micro-agglutination will be clear from a comparison of Fig. 1 and 2. Fig. 1 is a typical field from a purified preparation of the southern bean mosaic virus; Fig. 2 shows a mixture of this virus with its antibody. Similar microflocs are produced when the bushy stunt virus and its antiserum are mixed (Fig. 3). Micro-flocculation has also been observed with the tobacco mosaic virus protein and with other antigens when mixed with their anti-substances. Similar agglutinations were not seen when normal rabbit serum replaced specific serum in the mixtures. For example, in Fig. 4, which is taken of a mixture of bushy stunt virus and normal serum, virus particles are separate from one another in their enmeshing serum. The amount of virus in single microflocs on preparations such as those giving Fig. 2 and 3 often is no more than *ca.* 10^{-12} g.

In these photographs the background structure due to the collodion is often less sharply defined than usual. This apparent smoothing-over of detail in the substrate is probably due to an irreversible deposition of serum proteins over the entire surface of the preparation.

It is not so simple as might at first be imagined to measure accurately the apparent increase in particle size of the virus in these mixtures. The agglutinated particles of Fig. 2 and 3 are close to one another but their outlines are often diffuse and the irregularity of their vertical stacking prevents many determinations of particle-separation. Best results are obtained by seeking places where several particles are lined up in rows. Preliminary measurements on such rows indicate that the apparent separation of both agglutinated bean mosaic and bushy stunt virus particles is about twice that found in pure suspensions. More accurate studies are being made of mixtures containing different proportions of antigen in order to obtain more precise data.

Summary. Shadowed electron micrographs of mixtures of purified southern bean mosaic and bushy stunt viruses with their specific antisera show their spherical elementary bodies aggregated into microflocs in which the particle-separations seem to be about twice their normal values. More detailed electron micrographic study of these systems is underway.

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[8] Price, W. C., Williams, R. C., and Wyckoff, R. W. G., *op. cit.; Arch. Biochem.*, in press.

15210

The Effect of Lecithin Administered Intravenously.

GERALD K. ASHBY. (Introduced by Warren M. Cox, Jr.)
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It has been reported that various lecithins when administered by vein cause hemolysis,[1,2,3] leukocytosis,[4] changes in the fragility of the blood cellular components,[4,5,6] or death.[7] On the other hand, one investigator reports that regardless of the method of administration lecithin alone exerts no toxic action.[8] Because of the current interest in fat emulsions for parenteral use,[9,10,11] a brief study of the effect of lecithin when administered by vein has been made.

Experimental. Each sample of lecithin was prepared as a 7.0% colloidal solution in pyrogen-free distilled water and autoclaved at 10 pounds pressure for 15 minutes. The sterile solutions were rapidly injected into the marginal ear veins of normal rabbits in amounts sufficient to supply 1.75 g of lecithin per kilogram body weight. Table I shows the essential data obtained with 13 samples of lecithin. All but one of the injected rabbits died within 12 hours. These results strongly suggest that none of the tested lecithins is suitable for making fat emulsions for intravenous use.

Various methods of isolating lecithin from eggs were investigated, but the methods generally used for this purpose were not entirely successful in our hands.[12,13] The method finally adopted consisted of exhaustive extraction of powdered egg yolk with acetone to remove the fat, subsequent extraction with alcohol to obtain the crude lecithin, distillation of the alcohol under high vacuum, solution of the residue in ether and final precipitation of the lecithin with acetone. The precipitated lecithin was redissolved in ether, filtered and reprecipitated with acetone several times. Final traces of solvent were removed under high vacuum at low temperature. Yields by this method were 11% of the original weight of the egg powder.

Lecithin prepared in this manner and stored under high vacuum for varying lengths of time up to 228 days was injected into 14 normal rabbits without any apparent ill effects (Table II). The level of 1.75 g per kilo body weight was increased to 3.5 g per kilo and again without any ill effects, but 7.0 g proved to be fatal.

Samples of egg lecithin prepared and tested as above were then exposed to the air at room temperature and at 55°C for varying periods of time to determine if toxic properties would develop. As indicated in Table II, toxic properties did develop in as little as 14 days at 55°C and 47 days at room temperature. The toxicity which developed in 53 days at 55°C was profound, since both rabbits died in exactly 4 minutes after the infusions were begun. It was thought that perhaps this toxicity might be due to lysolecithin but efforts to purify the exposed sample from toxic substances by the differential solubility of lecithin

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[1] Schippers, J. C., *Biochem. Z.*, 1912, **41**, 158.

[2] Cruikshank, J., and Mackie, T. J., *J. Path. Bact.*, 1913, **18**, 99.

[3] Levin, B. S., *C. R. Soc. Biol.*, 1935, **119**, 80.

[4] Stassano, H., and Billon, F., *Compt. Rend.*, 1902, **134**, 318.

[5] Grönberg, A., and Lundberg, A., *Acta Med. Scand.*, 1928, **69**, 99.

[6] Grönberg, A., and Lundberg, A., *Acta Med. Scand.*, 1929, **72**, 291.

[7] Tompkins, E. H., *Am. Rev. Tuber.*, 1936, **33**, 625.

[8] Hanschmidt, E., *Biochem. Z.*, 1913, **51**, 171.

[9] Holt, L. E., Jr., Tidwell, H. C., and Scott, T. F., *J. Pediat.*, 1935, **6**, 151.

[10] McKibbin, J. M., Pope, A., Thayer, S., Ferry, R. M., Jr., and Stare, F. J., *J. Lab. and Clin. Med.*, 1945, **30**, 488.

[11] Dunham, L. J., and Brunschwig, A., *Arch. Surg.*, 1944, **48**, 395.

[12] Maltaner, F., *J. Am. Chem. Soc.*, 1930, **52**, 1718.

[13] MacLean, Hugh, and MacLean, Ida S., *Lecithin and Allied Substances*, 2nd ed., pp. 84-85, 1927.

TABLE I.
Lecithins from Various Sources.

Type of lecithin	Source*	Label description	Color	Consistency	Color of solution	No. rabbits injected	Lecithin g/kg/BW	Results
Egg	1	about 90%	dk. red	soft wax	dk. cream	2	1.75	Died in 4 and 105 minutes
”	2	—	” ”	” ”	” ”	1	1.75	Died during night
”	3	pure	red. bk.	” ”	brown	1	1.75	Died in 22 minutes
”	4	practical	brown	” ”	”	1	1.75	Died in 30 minutes
”	5	pure	dk. brown	” ”	”	1	1.22	Died during injection
Soybean	6	fat free phosphatides	white	granular	white	5	1.75	Died within 8 hrs†
”	6	commercial	amber	oily wax	”	1	1.75	Died during night
”	6	edible	lt. amber	heavy oil	”	1	1.75	” ” ”
”	6	”	dk. amber	” ”	cream	1	1.75	Died in 4 hours
Animal	5	90%	brown	wax	brown	1	1.75	Died during night
Vegetable	5	60% or more	lt. amber	heavy oil	white	1	1.75	” ” ”
”	5	60% ” ”	dk. amber	” ”	cream	1	1.75	” ” ”
”	5	app. 75%	lt. amber	hard wax	white	1	1.75	Complete paralysis recovered in 1 wk

* (1) Merck & Co.; kindly supplied by Dr. L. Emmett Holt, Jr.; (2) Difco Laboratories, Inc.; (3) Coleman and Bell Company; (4) Eastman Kodak Co.; (5) Pfanstiehl Chemical Company; (6) American Lecithin Company; kindly supplied for research by Dr. A. Scharf.

† One died in 7 min.

TABLE II.
Experimental Lecithin Preparations.

Method of preparation or treatment	Color of lecithin	Color of solution	No. rabbits injected	Lecithin g/kg/BW	Results
Acetone ppt'd from ether solution	amber	white	14*	1.75	No apparent ill effects
” ” ” ” ”	”	cream	1	3.5‡	” ” ” ”
” ” ” ” ”	”	”	1	7.0‡	Died during night
Exposed to air at room temp. 47 days	dk. red	brown	2	1.75	One died during the night; one no apparent ill effects
” ” ” ” ” ” 86 ”	brown	”	2	1.75	” ” ” ” ” ” ” ” ” ”
” ” ” at 55°C 14 days	dk. red	”	2	1.75	One no apparent ill effects; one died during week-end, 72-120 hours
” ” ” ” ” 53 ”	black	dk. brown	2	0.625	Both died 4 min. after start of inj.
Cadmium chloride pptn.[12]	amber	white	1	1.75	Died during night
Cadmium chloride pptn.†	”	”	1	1.75	No apparent ill effects

* Several of these rabbits were injected 2 or more times.

† Cadmium removed by pptn. with H_2S.

‡ In 14% solution.

and lysolecithin in ether[14] were unsuccessful.

Chemically speaking, the purest lecithin has been prepared through the cadmium salt.[12] This procedure did give a very light-colored and attractive preparation, but a fat emulsion prepared with it was promptly fatal to a dog and the lecithin itself was fatal to a rabbit (Table II). The toxicity of lecithin prepared in this manner was found to be due to traces of cadmium which could be removed by treatment with hydrogen sulphide.

Summary. Thirteen samples of commercial lecithin were fatal to rabbits when administered intravenously at a level of 1.75 g per kilo body weight.

A method for preparing lecithin from egg yolk is described and it is shown that lecithin so prepared is harmless to rabbits when given by vein even at a level of 3.5 g per kilo body weight. Lecithin so prepared will acquire toxic properties if exposed to the air. Elevated temperatures accelerate this process. If the lecithin is stored under high vacuum, toxic properties will not develop within at least 228 days.

Egg lecithin prepared through the cadmium salt may contain sufficient quantities of cadmium to produce cadmium poisoning.

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[14] Fiori, A., *Biochim. terap. sper.*, 1930, **17**, 267.

15211

In vitro Susceptibility of *Histoplasma capsulatum* to Therapeutic Agents.

John H. Seabury and Dorothy Artis. (Introduced by H. A. Davis.)

From the Department of Medicine, University of Michigan, and the Division of Hospital Laboratories, University Hospital, Ann Arbor, Mich.

During treatment of 3 cases of histoplasmosis in the early months of 1945, it was decided to carry out *in vitro* susceptibility studies on the yeast phase of *Histoplasma capsulatum.*

Methods. Five-day-old cultures of *Histoplasma capsulatum* in heart-brain infusion broth (Difco) were used as the test organism.

The drugs tested were neoarsphenamine, neostam, sulfadiazine, sulfathiazol, stilbamidine.* Two antibiotics, streptomycin* and penicillin were likewise tested. The drugs were added to the media, before inoculation of the organism, in the following mg% concentrations: 100, 50, 25, 20, 10, 1, 0.1, 0.01, and 0.001.

The streptomycin was employed in the same manner, but in concentrations of 2500, 1250, 750, 250, 125, 100, 75, 50, 25, 12.5, 10, 7.5, and 5 units per ml. Penicillin was used in concentrations of 10, 5, and 1 units per ml.

Culture tubes of heart-brain infusion at pH 7.4, containing the desired concentrations of drugs in 10 ml, were inoculated, in duplicate, with 0.5 ml of a 5-day broth culture of the yeast phase of the test organism and incubated at 37°C. These cultures were examined for growth at the end of 7 days of incubation. Similar tests were performed in which the amount of inoculum was reduced to 0.2 ml of the 5-day culture of *Histoplasma capsulatum.* Each of these series, in duplicate, was repeated once with identical results.

A control series, in which drug-free culture medium was inoculated with the same quantity of inoculum as used in the drug series, was performed with each experiment.

The cultures were examined at the end of 7 days, and subcultures were made from all tubes which did not show growth or which were very cloudy. In tests using neoarsphenamine, stilbamidine, and streptomycin, some precipitate was formed when these agents were added to the heart-brain infusion broth. In the case of streptomycin, this was believed to be due to lipoid substances present in the streptomycin. In the case of stilbamidine,

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* The stilbamidine and streptomycin used were kindly supplied by Merck and Co.

TABLE I.
In Vitro Effect of Various Concentrations of Therapeutic Agents on *Histoplasma capsulatum.*

Concn. mg%	100	50	25	20	10	1	0.1	0.01	0.001	*Control
Neoarsphenamine	0	+	+	+	+	+	+	+	+	+
Neostam	+	+	+	+	+	+	+	+	+	+
Sulfadiazine	+	+	+	+	+	+	+	+	+	+
Sulfathiazole	+	+	+	+	+	+	+	+	+	+
Stilbamidine	0	0	0	0	0	+	+	+	+	+

Concn. units/ml	2500	1250	750	250	125	100	75	50	25	12.5	10	7.5	5	1
Streptomycin	+	+	+	+	+	+	+	+	+	+	+	+	+	
Penicillin											+		+	+

0 No growth.
+ Growth.
* Control tube contained no drug and was always positive.

this was due to the sodium chloride present in the infusion broth, inasmuch as it is known that sodium chloride will produce crystallization of stilbamidine. The cause of the precipitate when neoarsphenamine was used is not known, but is probably related to the colloid-like properties of neoarsphenamine. This factor of precipitation undoubtedly reduced the amount of effective therapeutic agent present. At the time, it was impossible to determine the amount of each agent actually in solution.

Results. The results of these experiments are presented in Table I. On the basis of these experiments, it was found that neoarsphenamine in a concentration of 100 mg%, and stilbamidine in concentrations from 10 to 100 mg%, prevented the growth of *Histoplasma capsulatum.* As previously stated, the actual amount of neoarsphenamine and stilbamidine in solution was less than the tabulated concentrations.

The use of sulfadiazine, sulfathiazole, and neostam as well as streptomycin and penicillin exerted no inhibitory effect in the amounts used.

15212

Effect of Large Doses of Streptomycin and Influenza Viruses on Chick Embryos.

ALFRED L. FLORMAN, ALICE B. WEISS, AND FRANCIS E. COUNCIL.
(Introduced by V. E. Levine.)

From the Virus Section of the Ninth Service Command Laboratory, Presidio of Monterey, California.

Search for an effective therapeutic agent against viral infections has been stimulated by the success attained in treating bacterial diseases with sulfonamides and antibiotics. However, since the problem is complicated by the intracellular growth of viruses, it is perhaps not remarkable that the results up to the present time have been almost consistently negative.[1] Recently a small quantity of streptomycin[2,*] became available to us. Because of the effectiveness of this agent against a number of bacteria which have been resistant to previously studied antibiotics and chemotherapeutic drugs, and its low toxicity for the

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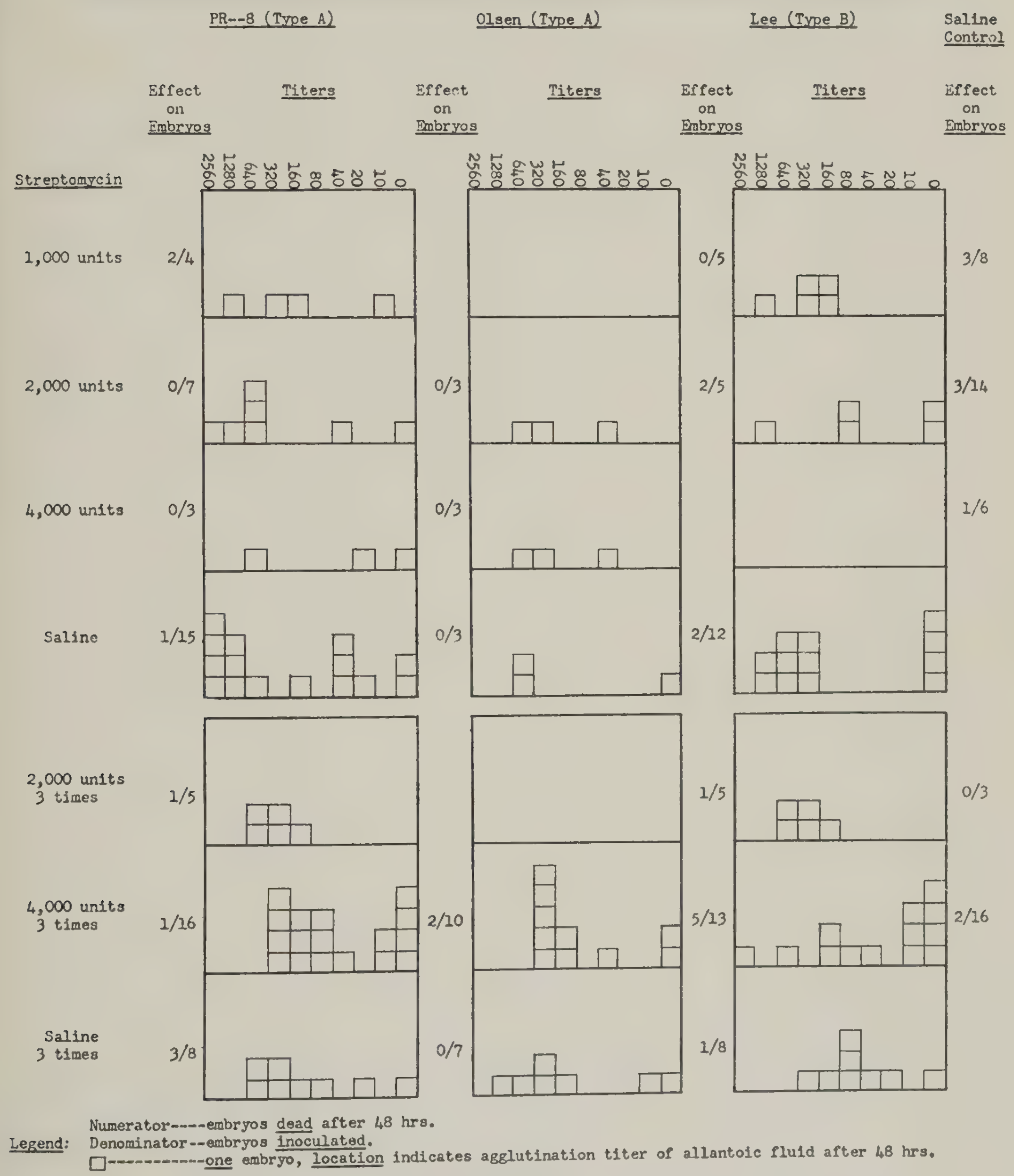
[1] Jones, D., Beaudette, F. R., Geiger, W. B., and Waksman, S. A., *Science,* 1945, **101**, 665.

[2] Schatz, A., Bugie, E., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 66.

* The streptomycin was supplied by Merck and Company through the courtesy of Dr. D. F. Robertson. It consisted of remnants of lots No. 199, 209, 212.

CHART I

Inability of Streptomycin to Prevent Infection of Embryonated Chick Eggs with Influenza Strains



Legend: Numerator----embryos dead after 48 hrs.
Denominator--embryos inoculated.
☐----------one embryo, location indicates agglutination titer of allantoic fluid after 48 hrs.

chick embryo,[3] a laboratory trial against viruses which would grow in the chick embryo seemed warranted.

Method of Study. The allantoic cavities of 11- to 13-day-old embryonated chicken eggs were inoculated with various strains of influenza combined with varying amounts of streptomycin. The PR-8 and Olsen strains were used as representative of Type A and the Lee strain as an example of Type B influenza.[†] Each embryo received an original total inoculum of 0.15 cc; of this 0.05 cc was a 1:1000 dilution of infected allantoic fluid, and 0.10 cc was either saline or the antibiotic reconstituted and diluted with sufficient saline to give the desired concentration. The virus and streptomycin were mixed immediately before inoculation. In those instances where more than one "dose" of streptomycin was given, the subsequent injections were made in 0.15 cc quantities within the first 24 hours. In the interim, the shellholes were sealed with a small tent of Scotch tape. The eggs were incubated at 36° to 37°C for 48 hours. After this period the allantoic fluids were collected and their hemagglutinating titers for chicken red cells were determined.[4]

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[3] Jones, D., Metzger, J. J., Schatz, A., and Waksman, S. A., *Science*, 1944, **100**, 103.

Results of Study. The results of several experiments are summarized in Chart I.[‡] From this it is apparent that:

1. A total of 12,000 units given over a period of 24 hours failed to make any significant difference in the multiplication in the chick embryo of the PR-8, Olsen, and Lee strains of influenza. This represents a total dosage of approximately 200,000 units per kilo of egg weight per 24 hours. It may be contrasted with the 150 and 300 units of streptomycin which protect chick embryos against *Shigella Gallinarum* and *Brucella abortus* infections.[3]

2. Despite the very large amounts of streptomycin administered in these experiments there was no evidence of any lethal effect on the developing embryo. The low toxicity of this new agent is noteworthy.

[†] The influenza strains were furnished by Dr. M. D. Eaton.

[4] Florman, A. L., and Crawford, J. P., *Am. J. Med. Sci.*, 1944, **208**, 494.

[‡] The chart was prepared by Cpl. Fred W. Trader and photographed by the U.S.A. Signal Corps.

15213 P

Protection of Mice Against Lethal Action of Gonococcal Endotoxin by Penicillin.*

C. PHILLIP MILLER AND ALDEN K. BOOR.

From the Department of Medicine and the A. B. Kuppenheimer Foundation, University of Chicago.

We have reported[1] that large subcutaneous doses of penicillin repeatedly administered exert a considerable degree of protection against the toxicity of sterile meningococcal endotoxin as measured by its lethal action in mice and rabbits. Neter[2] claimed that penicillin mixed with tetanus toxin did not protect mice from death. According to Ercoli, Lewis, and Moench[3] large doses of penicillin neutralized diphtheria toxin *in vitro,* but had no effect in guinea pigs on the lethal action of incompletely neutralized diphtheria toxin. Little other investigation has been directed toward the effect of penicillin on the action of toxic bacterial substances. Carpenter and his co-workers[4,5,6] reported detoxification by sulfanilamide and sulfapyridine of the "toxins" of gonococcus and other bacteria.

The crude endotoxin used in these experiments was prepared from 5 strains of gonococcus grown for 18 hours on an agar medium[7]

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* Aided by a grant from the John and Mary R. Markle Foundation.

[1] Boor, Alden K., and Miller, C. Phillip, *Science,* 1945, **102**, 427.

[2] Neter, Erwin, *J. Inf. Dis.,* 1945, **76**, 20.

[3] Ercoli, N., Lewis, M. N., and Moench, Lucille J., *J. Pharm. Exp. Ther.,* 1945, **84**, 120.

[4] Carpenter, C. M., Hawley, P. L., and Barbour, G. M., *Science,* 1938, **88**, 530.

[5] Barbour, Gerald M., and Carpenter, Charles M., Am. Assn. Adv. Sci., Pub. No. 11, 1939, 114.

[6] Carpenter, Charles M., and Barbour, Gerald M., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 354.

[7] Boor, Alden K., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 22.

TABLE I.

Effect of Penicillin on the Lethal Action of Gonococcal Endotoxin in Mice.

Gonococcal endotoxin	Dose, ml	Control mice Died/injected	Penicillin treated mice Died/injected
From strain 362	0.4	11/12	6/12
	0.1	8/12	2/12
From strain 354	0.5	10/10	6/10
	0.25	8/10	2/10
	0.1	7/10	0/10

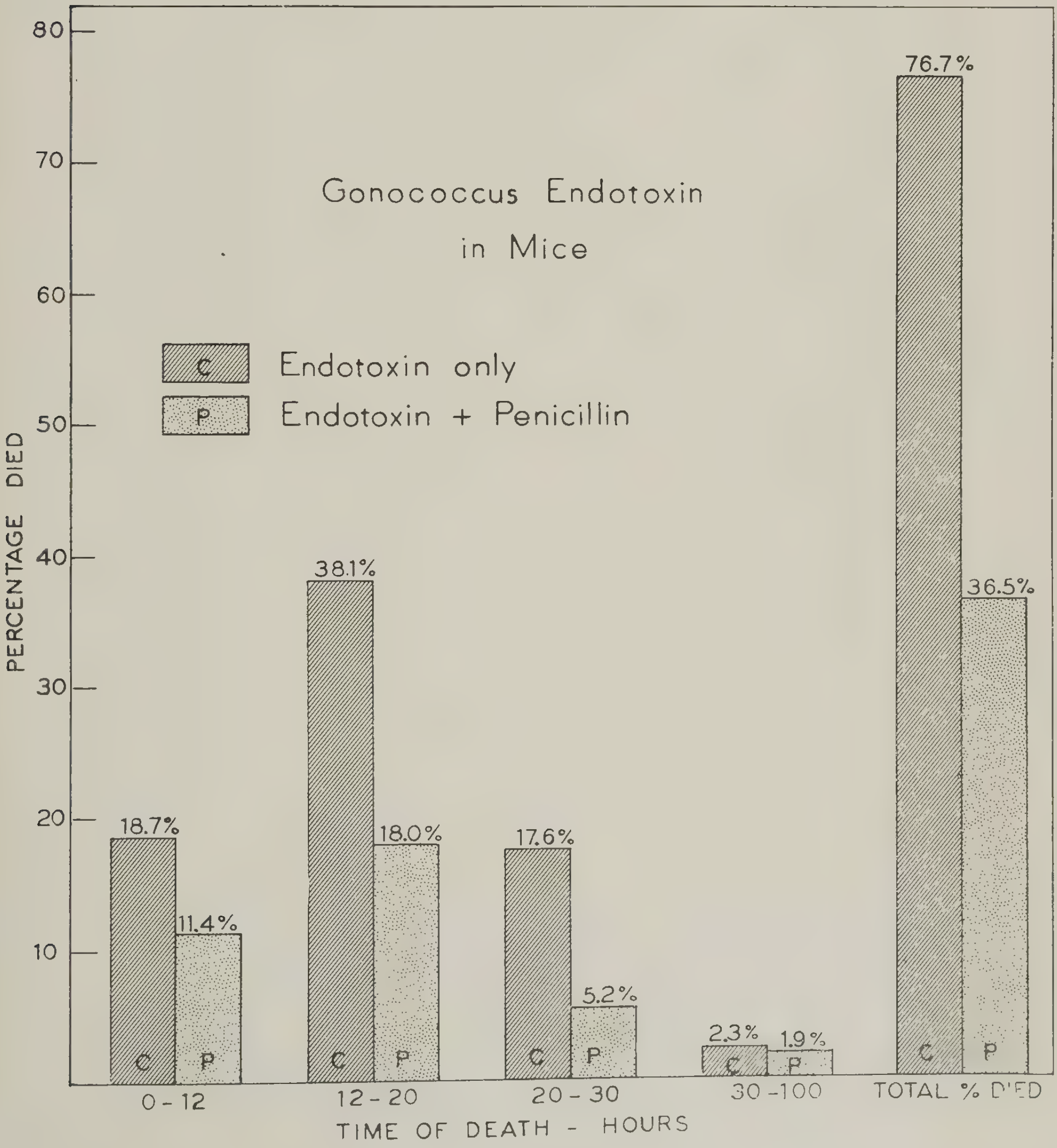

FIG. 1.

containing casein-digest and cystine. The organisms were washed off with saline, centrifuged, resuspended, and recentrifuged three times to free them from medium. The packed cells were finally suspended in water, adjusted to pH 8.0 and kept about 6 hours at room temperature and 14 hours at 4°C. Next the preparation was neutralized and sterilized by heating at 60°C for 30 minutes. Its concentration was adjusted to about 1.0% solids. All injections of endotoxin were made intraperitoneally in 18- to 20-g white mice. The toxicity of each preparation was determined by preliminary tests in batches of 5 or 6 mice for each trial dose.

The penicillin[†] used was the ordinary commercial product of several manufacturers. The mice received 8 subcutaneous injections of penicillin of 1,500 units each in 0.15 cc of water. These were usually given as follows: at 90- and 45-minute intervals before the endotoxin and at 1, 3, 5, 9, 20, and 24 hours after the endotoxin. Variations in total quantity and number of doses of penicillin were tried but no improvement was found in the amount and method of administration. Mice were used in groups of 8 to 15 for each quantity of endotoxin in the control group as well as in the group receiving penicillin. Total number of deaths was determined for a period of 90 hours after injection of the endotoxin.

In most of the experiments graded doses of endotoxin were used. Representative of some of the more striking experiments are the two summarized in Table I. In each group injected with a given dose of endotoxin, more deaths occurred among the controls than among those treated by penicillin.

Some experiments showed better protection than others, but in every one fewer of the treated mice died than of the controls. The combined results of all the series, totaling 176 control mice and 210 penicillin treated, are summarized in Fig. 1, which shows the rates of death as well as final percentages.

The difference in survivals and deaths as shown on this chart may be considered significant. Whereas 76.7% of all the control mice died, only 36.5% of those receiving the penicillin treatment died.

During the first 12 hours after endotoxin injection, 18.7% of the controls and 11.4% of the penicillin-treated mice died—some as early as 3 hours. From 12 to 20 hours the highest mortality occurred—38.1% of the controls and 18% of those treated with penicillin.

Other substances such as saline and tryptic digest of casein repeatedly injected in place of the penicillin failed to influence the lethal action of the endotoxin.

Summary and Conclusions. Large doses of penicillin repeatedly administered by subcutaneous injection protected a significant proportion of mice against the lethal action of a crude preparation of gonococcal endotoxin.

[†] The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for experimental investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

15214

Action of Anterior Pituitary on Sertoli Cells and on Release of Toad Spermatozoa.

E. DE ROBERTIS, M. H. BURGOS, AND E. BREYTER. (Introduced by Vergilio G. Foglia.)
From the Institute of General Anatomy and Embryology, Faculty of Medicine, Buenos Aires, and Institute of Biology and Experimental Medicine.

Houssay and Lascano Gonzalez[1] found that in the testis of toads, in which anterior pituitary glands were implanted, spermatozoa were released into the lumen of the seminiferous tubules. Rugh[2,3] confirmed this ob-

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[1] Houssay, B. A., and Lascano Gonzalez, J. M., *Rev. Soc. Arg. Biol.*, 1929, **5**, 77.

[2] Rugh, L. R., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 418.

[3] Rugh, L. R., *J. Exp. Zool.*, 1939, **80**, 81.

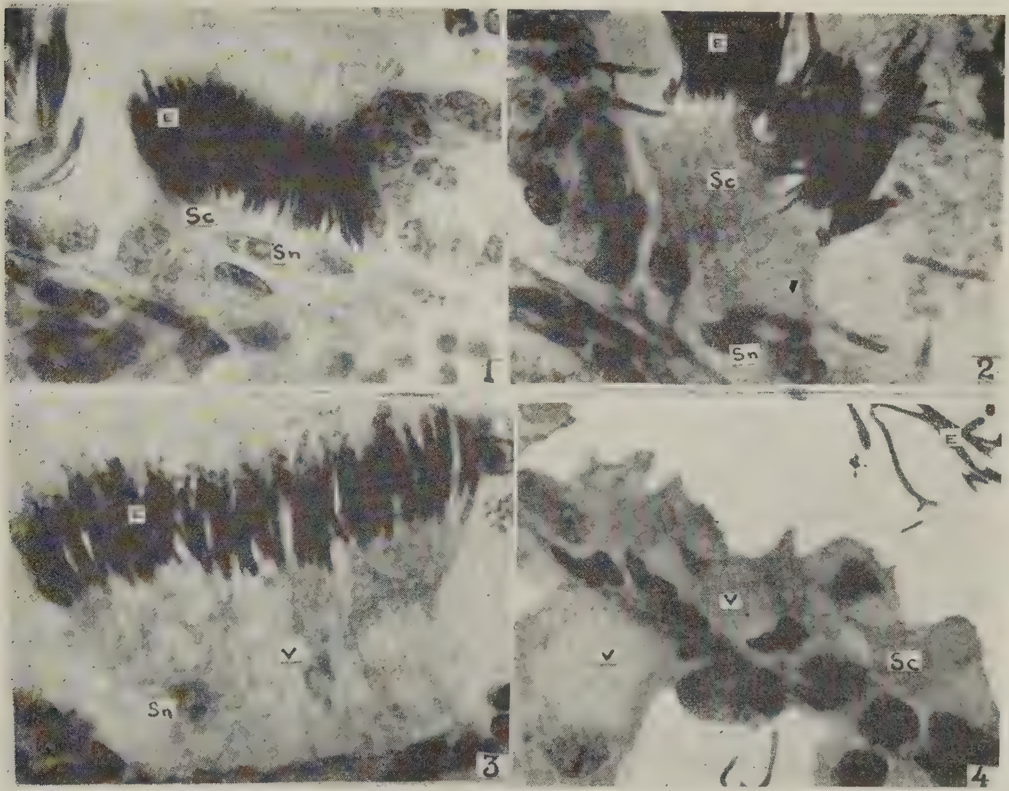

Fig. 1-4. Testis of *Bufo arenarum* Hensel. Hematoxylin-eosin. Ob. 90x, oc. 6x, Cam. length 60 cm. Sc, Sertoli cell; Sn, Sertoli nucleus; E, spermatozoon; v, vacuole.

FIG. 1. Normal control. Spermatozoa tightly clustered upon Sertoli cells. Fix.: Zenker-formol.

FIG. 2. Thirty minutes after the injection of the pituitary extract. Cytoplasm of Sertoli cells is greatly increased, showing a fine vacuolization and small eosinophilic granules. Fix.: Bouin's fluid.

FIG. 3. One hour after injection. Cytoplasm of the Sertoli cells is more hypertrophic and shows big vacuoles. Fix.: Bouin's fluid.

FIG. 4. Three hours after injection. Spermatozoa are completely liberated and released into the lumen. Sertoli cells appear highly vacuolized and with the apical border torn. (Lumen slightly retouched.)

servation finding that the injection of pituitary extract produces, in the testis of hibernating frog, the release of the spermatozoa, normally attached to the Sertoli cells. The author gives no explanation of the mechanism by which the spermatozoa are released into the lumen, but suggests that: "Possibly smooth muscle fibers are involved in the expulsion of spermatozoa from the tubules" (Rugh[3]).

In order to investigate the actual mechanism of this release, toads (*Bufo arenarum* Hensel) in sexual rest (end of summer) were injected intravenously with a total extract of 3 anterior lobes of the pituitary gland of male toads. Thirty minutes, one, 2, 3, and 5 hours after the injection, the testes were fixed in Bouin or in Zenker-formol fluids; and also in the fixative of Champy, followed for 7 days by immersion in osmic acid at 38°C. Testes of normal control animals showed almost all mature spermatozoa clustered about their Sertoli cells (Fig. 1). Thirty minutes after the injection of the pituitary extract some of the spermatozoa have been liberated. The Sertoli cells showed big increase and vacuolization of cytoplasm (Fig. 2). After one hour, clear hydropic vacuoles were more conspicuous and also some acidophil droplets were found (Fig. 3). The vacuoles have the tendency to aggregate and seem to push the spermatozoa away from the tubule wall. Three hours after the injection almost all mature spermatozoa have been released. Most of the Sertoli cells appear enormously vacuolized and with the apical border completely irregular, due to the rupture of some vacuoles (Fig. 4). During this process of vacuolization, the Golgi apparatus appears fragmented and situated close to the vacuoles.

The observations recorded here show that possibly the release of the spermatozoa of the toad, under the influence of the pituitary gland, is due to the intense changes produced in the Sertoli cells, which lead to the rupture and disintegration of the apical part of their cytoplasm.

15215 P

A Method for Removal of Adult *S. mansoni* from Experimentally Infected Rabbits.

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Herein is described a method for the removal of adult *S. mansoni* worms from experimentally infected rabbits. The method has been used with good results in rabbits although there is no apparent reason why it could not be applied to other experimental animals susceptible to schistosomiasis.

The animal is tied to a board and a large dose (100 mg) of Heparin (Abbott—10 mg per cc) in 30 to 40 cc of physiological saline is injected very slowly into the heart or intravenously. Between 15 to 30 minutes are allowed to elapse at which time the animal is sacrificed in any desired fashion, the abdomen opened and the portal vein exposed. An 18-gauge needle with attached 50-cc syringe is inserted into the portal vein with the point of the needle directed towards the liver. The heparinized blood in the portal vein is aspirated while the syringe and needle are rotated to direct the bevel of the needle toward the worms which will be seen flowing into the syringe with the blood. About 15 to 20 cc can thus be aspirated before the portal vein collapses. An additional 10 to 15 cc of blood can be aspirated by exerting a gentle squeezing pressure on the lobes of the liver—this will force more blood into the portal vein, and the liver will be seen to blanch slightly. The syringe is disconnected from the needle and put aside without fear of the blood clotting. A clean dry syringe may be attached to the needle and additional aspirations carried out in the manner described.

If it is desired to keep the worms alive, the aspirated blood is poured into a solution of 0.5% saponin in physiological salt solution (0.85%)—this will lake the blood cells but have no apparent effect on the worms. If it is not necessary to keep the worms alive, the aspirated blood is poured into a large volume of tap water—this will lake the blood cells, and the dead worms will sink to the bottom of the vessel.

When no further aspirations can be done the needle is removed from the portal vein. The liver is removed as a unit by cutting and clamping the hepatic vein, portal vein, surrounding ligaments and adhesions and kept in saline for further recovery of worms. It is then placed on a dissecting board and the thin lips of the various liver lobes are cut off about ¼ inch from their edges. Using a 50-cc syringe, physiological saline is perfused through the liver by injecting the hepatic vein.

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* The authors wish to express their gratitude to Dr. P. Morales-Otero, Director, and to Dr. Jose Oliver-Gonzalez of the School of Tropical Medicine for their advice and encouragement during this work.

Additional worms will be seen in the perfusate exiting from the cut edges of the liver and from the portal vein. When no further hepatic vein perfusion yields any worms, the liver is divided into its separate lobes and these cut into smaller pieces about 3 to 4 inches square. The larger portal radicals may be perfused and additional worms obtained. By a gentle squeezing and shaking motion of the pieces of liver additional worms can be recovered. (The intact liver may be kept in saline overnight safely and the above procedure carried out with the same results as with livers done shortly after removal from the animal's body.)

The mesentery of the large and small bowel is stripped from its attachments, cut into small pieces and placed in saline. This is allowed to stand at ice-box temperature overnight, at which time all the worms remaining in the mesenteric venous system will have escaped and seen lying on the bottom of the container.

Experiments are being carried on to determine fully the advantages of this method over other methods described.

15216

Subtilin—Antibiotic Produced by *Bacillus subtilis.** II. Toxicity of Subtilin to Living Embryonic Tissue.†

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In a previous communication[1] it was shown that subtilin, an extract prepared from *Bacillus subtilis,* was antagonistic chiefly against Gram-positive bacteria. Two notable exceptions were *Neisseria catarrhalis* and *N. gonorrhoeae,* both Gram-negative but also antagonized by subtilin. Acid-fast organisms, including *Mycobacterium tuberculosis,* and a number of pathogenic higher fungi were also found to be susceptible to the antibiotic. The agent produced a bacteriostatic action in high dilution and a germicidal effect in greater concentration.

The present communication is concerned with the toxicity of subtilin to living embryonic chick heart tissue fragments cultivated *in vitro*. The procedures followed in performing the tests were the same as those previously reported.[2,3]

Experimental. The highest dilution of subtilin that killed embryonic chick heart tissue fragments in 10 minutes at 37°C was found to be 1:500 (Fig. 1). The highest dilution of subtilin that killed *Staphylococcus aureus* (F. D. A. strain) under the same conditions was 1:9750 (Table I). From these results a toxicity index may be calculated.

The toxicity index may be defined as the ratio of the highest dilution of subtilin killing embryonic chick heart tissue after an exposure period of 10 minutes at 37°C to the highest dilution killing *Staphylococcus aureus* under the same conditions. The results give a toxicity index of $500/9750 = 0.05$ (Table II). Under the conditions of the test, subtilin was found to be 20 times more toxic to *Staphylococcus aureus* than to embryonic chick heart tissue, a remarkably low figure. Theoretically, the smaller the toxicity index the more nearly perfect the germicide.

Unitage. The methods used for expressing the potencies of the various antibiotics are, in general, quite complicated and confusing. In order to simplify the method of designating

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* This investigation was aided by a grant from Eli Lilly and Company, Indianapolis, Ind.

† The subtilin preparation used in these experiments was kindly supplied by the Western Regional Research Laboratory, Albany, Calif.

[1] Salle, A. J., and Jann, Gregory, J., Proc. Soc. Exp. Biol. and Med., 1945, **60**, 60.

[2] Salle, A. J., McOmie, W. A., Shechmeister, I. L., and Foord, D. C., Proc. Soc. Exp. Biol. and Med., 1938, **37**, 694.

[3] Salle, A. J., McOmie, W. A., Shechmeister, I. L., and Foord, D. C., *J. Bact.*, 1939, **37**, 639.

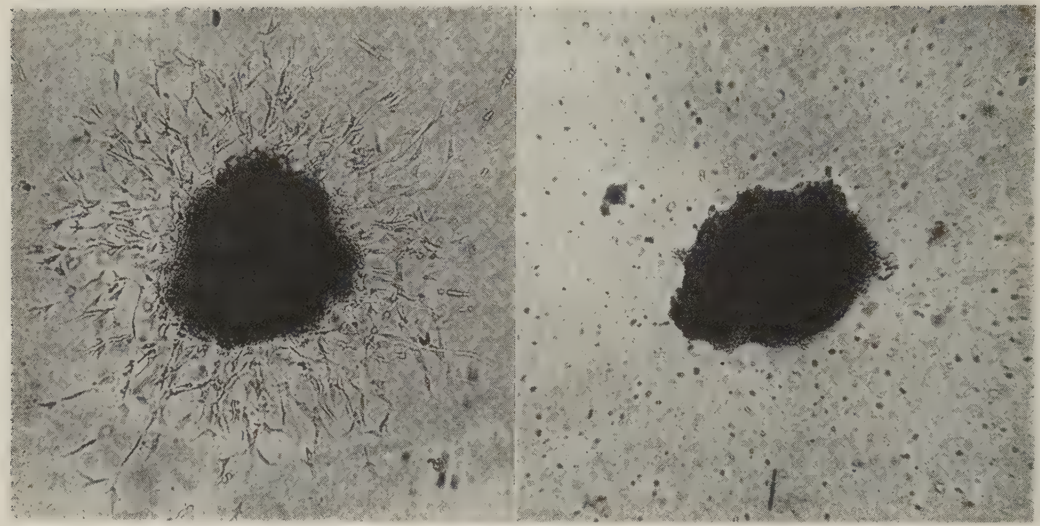

FIG. 1.
Left, fragment of chick heart tissue exposed to a sublethal concentration of subtilin. Note new growth of fibroblasts. Right, fragment exposed to a lethal concentration of subtilin.

TABLE I.
Killing Dilutions of Subtilin for Chick Heart Tissue Fragments and *Staphylococcus aureus.*

Tissue fragments		*Staphylococcus aureus*	
Dilution	Growth	Dilution	Growth
1:100	—	1:9000	—
1:200	—	1:9250	—
1:300	—	1:9500	—
1:400	—	1:9750	—
1:500	—	1:10,000	+
1:600	+	1:10.250	+
1:700	+	1:10,500	+
1:800	+	1:10,750	+
Control	+	Control	+

TABLE II.
Toxicity Index of Subtilin.

Killing dilution		
Tissue (A)	*Staphylococcus aureus* (B)	Toxicity index A/B
1:500	1:9750	0.05

the strength of subtilin as much as possible, a unit is defined as that amount present in 1 cc of the highest dilution (expressed in mg) capable of killing *Staphylococcus aureus* in 10 minutes as 37°C (F.D.A. phenol coefficient method). For example, 5 cc of a 1:9750 dilution of the preparation used, when mixed with 0.5 cc of a nutrient broth culture of *Staphylococcus aureus,* completely destroyed all organisms in 10 minutes at 37°C. One cc of this dilution contains approximately 0.1 mg of this preparation of subtilin. Therefore, 0.1 mg contains 1 unit of subtilin. The weight of subtilin containing 1 unit will vary depending upon the purity of the product.

The 24-hour culture of *Staphylococcus aureus* was standardized in a photoelectric colorimeter to contain the same density as a No. 7 McFarland Nephelometer Standard.

Summary. Subtilin, an antibiotic extracted from *Bacillus subtilis,* was found to be antagonistic chiefly against Gram-positive organisms, including *Mycobacterium tuberculosis* and other acid-fast bacteria. Two notable exceptions were *Neisseria catarrhalis* and *N. gonorrhoeae,* both Gram-negative but also antagonized by subtilin. The antibiotic showed an extremely low toxicity for embryonic chick heart tissue fragments cultivated *in vitro.* Under the conditions of the test subtilin was approximately 20 times more toxic to *Staphylococcus aureus* than to chick heart tissue, a remarkably low tissue toxicity. A unit of subtilin is defined as the amount contained in 1 cc of the highest dilution capable of killing *Staphylococcus aureus* in 10 minutes at 37°C (F.D.A. phenol coefficient method).

15217

Amyloidosis in Hamsters with Leishmaniasis.*

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During the course of an investigation of chemotherapeutic agents in experimental leishmaniasis, the development of anasarca in infected animals was first noted by R. O. Greep in the early stages of the experimental program in the Squibb Institute in New Brunswick, New Jersey. Later, routine histopathological examination as well as chemical investigation of the blood revealed the widespread presence of amyloid deposits and a reversal of the albumin-globulin ratio of the plasma proteins. In human kala azar, the presence of generalized edema is not infrequent and the alteration of the serum albumin-globulin ratio is characteristic; however, neither in clinical nor in experimental leishmaniasis have we been able to find a report of amyloidosis.[1] Nevertheless, it seems likely that the hamsters described in the note just published by Goodwin[2] suffered from amyloidosis. In his animals, as in ours, infection by *Leishmania* might be followed by edema, nephritis, proteinuria, and depression of the level of plasma proteins. The present investigation was undertaken to correlate the development of amyloidosis and the changes in the distribution of proteins in the plasma during the progress of leishmaniasis in hamsters. From the experimental results it was hoped that answers to two questions would emerge: (1) What is the temporal relationship between the deposition of amyloid and the alteration of the plasma protein concentration, and (2) Alterations in which organ, the liver or the kidney, are chiefly responsible for the reduction of the plasma albumin?

Materials and Methods. A single large group of hamsters (*Cricetulus auratus*), six weeks old, served as the experimental animals. They were maintained on pellets of Rockland rat diet ("D" free) supplemented with lettuce, carrots, and whole wheat bread. The animals were divided into two groups at random and at the beginning of the experiment the animals of one group were inoculated intraperitoneally with 20 mg of spleen in a saline emulsion which was obtained from 2 hamsters heavily infected with *L. donovani* (Khartoum strain.) The other group served as the uninfected control. At approximately two-week intervals, 5 representatives each from the control and the infected groups were placed in individual metabolism cages and the urine was collected for 24 hours.

Immediately after the termination of the urine collection, the animals were anesthetized with intraperitoneal evipal sodium (140 mg/kg body weight) and heparinized blood samples were obtained by heart puncture. The animals were then sacrificed; the tissues removed for histological examination were fixed either in Zenker's solution without acetic acid or in Vandegrift's dehydrating fixative.

Owing to the small size of the plasma sample for the determination of non-protein nitrogen, it was necessary to determine this nitrogen fraction by a Nessler procedure which was adopted for all nitrogen determinations. The solutions of protein or non-protein nitrogen were first digested in Pyrex Nessler tubes in the presence of 1 ml of 19 N sulfuric acid; oxidation was completed by a drop or two of 30% hydrogen peroxide. The digested material was diluted with water to 35 ml and 15 ml of Nessler's reagent made according to the directions of Folin[3] were added. The final

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* This work has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.

[1] Stitt's *Diagnosis, Prevention, and Treatment of Tropical Diseases* edited by R. P. Strong, Philadelphia, 1943.

[2] Goodwin, L. G., *Nature*, 1945, **156**, 476.

[3] Folin, O., *Laboratory Manual of Biological Chemistry*, New York, 1934.

TABLE I.

Plasma Proteins, Amyloidosis and Edema in Hamsters with Experimental Leishmaniasis.

Days after Infection		Albumin (g%)	S.D.	Globulin (g%)	S.D.	A/G Ratio	S.D.	Edema	Amount of amyloid in Kidneys (glomeruli)	Adrenal cortex	Spleen	Liver	No. of animals
18	Normal	2.76	0.16	2.06	0.17	1.35	0.18	0	0	0	0	0	3
	Infected	2.12	0.62	3.06	0.61	0.75	0.38	0	0	0	0	0	5
32	Normal	3.09	0.35	2.19	0.43	1.45	0.31	0	M.*1/5	M.1/5	M.1/5	M.2/5	5
	Infected	3.07	0.45	2.76	0.23	1.11	0.20	0	M.1/5	M.2/5	M.1/5	M.1/5	5
46	Normal	2.34	0.33	2.56	0.21	0.92	0.16	0	0	0	0	0	5
	Infected	1.09	0.66	2.72	0.40	0.40	0.22	+2/5	+++5/5	++5/5	+4/5	+4/5	5
60	Normal	3.17	0.17	1.98	0.19	1.60	0.17	0	M.1/5	0	M.1/5	M.1/5	5
	Infected	0.48	0.21	2.22	0.58	0.22	0.07	+++3/3	++++3/3	+++3/3	+3/3	+3/3	3
77	Normal	2.46	0.26	2.26	0.24	1.11	0.06	0	0	0	0	0	3
	Infected	0.61	0.17	2.34	0.29	0.26	0.05	+++4/4	++++4/4	+++4/4	+4/4	+4/4	4

* M = Minimal.

estimates were made in a Beckman photoelectric spectrophotometer at a wave length of 400 mμ. The useful range of amounts of nitrogen per sample was about 0.02 to 0.15 mg. The blank solution was similarly made from reagents alone; with known amounts of nitrogen added as a solution of pure $(NH_4)_2SO_4$, there was a straight-line relationship between optical density and nitrogen-concentration. The method of Howe[4] was employed to separate plasma globulin and thus to determine the concentration of albumin and globulin in plasma. The non-protein nitrogen of urine and plasma was estimated in filtrates containing, after suitable dilution with water, 5% trichloroacetic acid. The histological examinations were made independently by one of us (WJP) and it was not until the experiment was completed that the results of the pathological examination and the chemical changes in the blood were compared. Paraffin sections of the tissues were made and stained with hematoxylin and eosin, Giemsa's stain, or Congo Red which was sometimes combined with Wilder's reticulum stain.

Results. The principal gross pathological findings in infected hamsters were splenomegaly, enlarged lymph nodes, pale kidneys and anasarca. The gross renal change and the anasarca were not evident until 46-60 days after inoculation. There were no significant gross changes in the tissues of the normal controls. The microscopic alterations owing to leishmaniasis conformed to the description of other authors with the exception of the presence of amyloidosis which was found in all hamsters which had been inoculated with *L. donovani* at least 46 days previously. Small amyloid deposits were found in 3 of the 21 control animals; the quantities present were minimal.

The incidence of the amyloidosis is summarized with the other data in Table I. Amyloid was deposited chiefly in the renal glomeruli, the adrenal cortex, the spleen and the liver. Its appearance and distribution were characteristic of "secondary" amyloidosis of man and animals. No intracellular amyloid was identified. The amyloid of the

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[4] Howe, P. E., *J. Biol. Chem.*, 1921, **49**, 93.

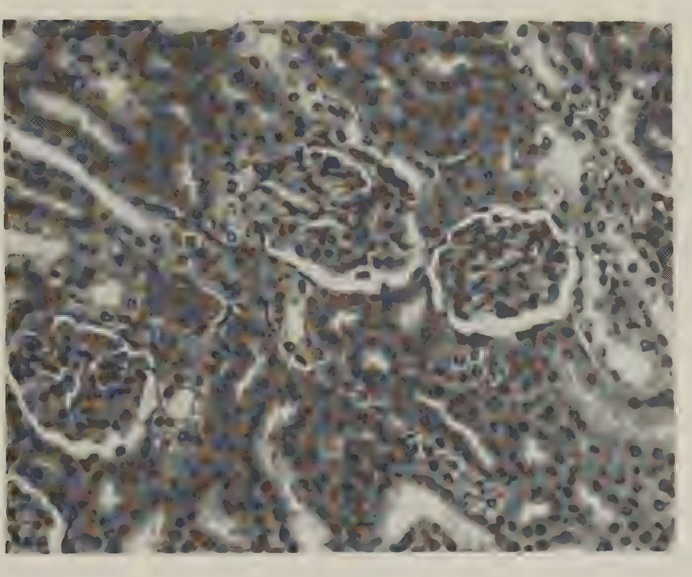

Fig. 1.
Kidney of normal hamster. Hematoxylin and eosin. × 275.

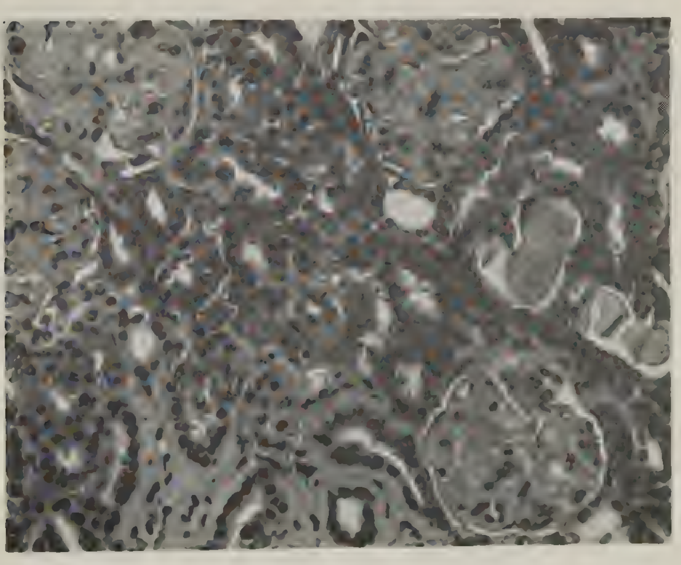

Fig. 2.
Kidney of hamster with amyloidosis 77 days after inoculation of *L. donovani.* Note extensive replacement of glomeruli by amyloid deposited between endothelium and epithelium. The tubular casts are also typical. Amyloid deposited about the tubules and extending into the interstitial tissue is an infrequent finding illustrated in this section. Hematoxylin and eosin. × 275.

kidneys was deposited in the glomeruli and appeared to replace three-fourths or more of this structure in the late stages of the disease. (Leishmania were rarely found in the kidney.) The glomerular amyloid accumulated between the capillary endothelium and the capillary basement membrane; it was never found external to the latter. Trivial deposits of amyloid were observed between the epithelium and the basement membrane of the tubular epithe-

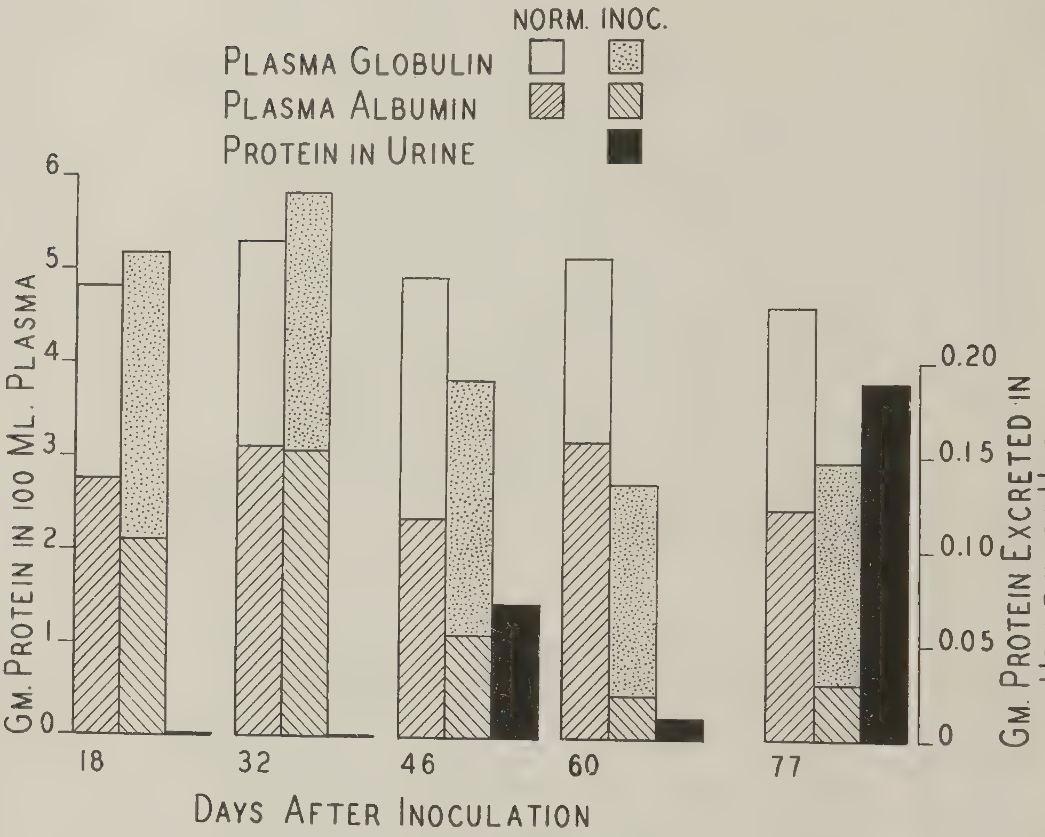

FIG. 3.
The concentration of plasma proteins in normal and infected hamsters. Also shown is the total urinary protein excretion in infected hamsters.

lium. Hyaline droplets or casts could be found in the tubules of the kidneys with severe amyloidosis. (Fig. 1 and 2.) In the cortex of the adrenals there was likewise bilateral deposition of amyloid which in some cases replaced more than half of the normal tissue. The early deposits in the adrenal cortex were clearly between the capillary endothelium and the basement membrane. The amyloid of the spleen was deposited about the widely separated malpighian corpuscles and, to a less extent, in the ring of pulp surrounding the spherical masses of phagocytic cells containing parasites. In the liver, amyloid was first found beneath the endothelium of the hepatic and portal veins from which it occasionally spread into the parenchyma. Deposits sometimes occurred around parasite-filled phagocytic cells. The amounts of amyloid in spleen and liver were always small.

Table 1 also presents the quantitative data on the plasma albumin and globulin and the A/G ratio at intervals after the inoculation of the animals. The standard deviations are also tabulated to indicate the range of the observations and their distribution about the mean. It is to be noted that 46 days after the infection there is a significant decrease in the plasma albumin concentration of the infected animals as compared with the controls. The globulin concentration is not demonstrably different in the two groups and as a result the A/G ratio is significantly lower in the infected animals. It is also to be noted that at this time the deposition of significant amounts of amyloid makes its first appearance and that it is found to be most abundant in the glomeruli of the kidney. Between the 46th day and the 60th day the decrease in plasma albumin concentration in the infected animals continues precipitously. In the next interval of 17 days there is no further change in the plasma albumin concentration of the infected animals. During the entire time when the striking fall in plasma albumin concentration is occurring, there is no significant deviation of the plasma globulin level in the infected animals as compared with the normal group. Amyloidosis is maximal at the 60th day and the distribution of amyloid is at all times more widespread in the kidney (glomeruli) than in the liver. The presence of edema is correlated with the hypoalbuminemia as one would expect.

The plasma non-protein nitrogen concentration in the infected animals was not significantly different from the control in all the experiments except at 60 days. In this experiment the non-protein nitrogen concentration of the infected group was clearly above the level in the control animals.

Fig. 3 presents the results of the plasma protein studies graphically, and, in addition, summarizes the quantitative estimation of proteinuria. It can be seen that at no time did the control groups show any protein in the urine. In the infected animals, proteinuria made its first appearance after 46 days, when the first observation of hypoalbuminemia was made. Proteinuria was always observed subsequently. The available evidence suggests that the urinary protein was albumin; none could be precipitated in the presence of 1.5 M sodium sulfate.

Discussion. In another series of hamsters attempts to inoculate one strain of *L. infantum* by intraperitoneally-injected splenic emulsion did not produce infection or recognizable edema within the following 6-8 weeks. For this reason, normal spleen was not administered intraperitoneally to the control animals.

In all the experiments, the percentage of globulin in the plasma of the infected animals was higher than that of the normals; however, no statistical significance can be attached to these differences. (Because of the high percentage of globulin in the plasma of the infected hamsters of the first experiment, the A/G ratio was reduced.) There can be no doubt that in hamsters infected with *Leishmania donovani* 46 days or longer, there was a significant fall in the plasma albumin and in the A/G ratio in comparison with corresponding control animals. The highest probability that such a change could occur by chance was less than 0.01 (comparison of albumin-concentrations of experiment 3.) In all other comparisons of plasma albumin percentage in plasma or of the A/G ratio, the probability of chance occurrence was even lower—especially in the last 2 experiments. Albuminuria in the infected hamsters was first detected 46 days after infection when a significant lowering of plasma albumin was also first observed. The small amount of albumin excreted in the urine 60 days after infection is to be explained by the small quantity of urine excreted owing to excessively hot weather. All the protein in the urine may have been albumin since no globulin could be precipitated by warm sodium sulfate solution according to Howe's plasma method; however, no dogmatic statement in this regard can be made since the experimental conditions are seriously altered when urine instead of plasma is the protein solvent and the concentration of protein present is different from that of plasma.

The animals infected 46 days or longer, in contrast with the controls, had hypoalbuminemia, proteinuria, edema (characterized by anasarca in the last 2 experiments) and deposits of amyloid in the kidneys, adrenal cortex, spleen and liver. These changes occurred 46 days after infection. It is concluded that the primary change, amyloid deposition in the renal glomeruli followed later by a similar but slight change in the tubules, led to excessive loss of albumin in the urine with an associated reduction of plasma albumin concentration and lowering of the colloid osmotic pressure of plasma so that excessive amounts of fluid escaped into the tissues. It is not believed that failure of the synthesis of plasma albumin was responsible to any important extent for the lowering of the percentage of this protein in the plasma.

Summary. Forty-six days after successful inoculation of *L. donovani* into hamsters, there appeared edema associated with amyloidosis. There was marked anasarca at 60

and 77 days after infection with extensive deposition of amyloid in the glomeruli and the adrenal cortex. The hypoalbuminemia, which coincided with the edema and a proteinuria, is believed to have been caused by the impairment of glomerular function.

15218

Occurrence of Two Immunological Groups Within the Genus Listeria. Studies Based upon Precipitation Reactions.

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Thus far, attempts to establish the serological reactions and antigenic structure of microorganisms of the genus Listeria have been based upon agglutination and agglutinin absorption studies. Paterson[1] concluded that the antigenic structure permitted division of the genus into 4 types. He stated, "the bacteriological types do not bear any relation to the zoological species of the host or to the geographical distribution of the places of isolation." Schultz, Terry, Brice and Gebhardt[2] on the other hand found that among 11 different strains of *Listeria monocytogenes* only 2 serological groups occurred. The strains were derived from various animal sources, including man. On the basis of the agglutination reaction Julianelle and Pons[3] divided 8 strains into 2 types which they designated Type I and Type II. Type I represented strains isolated from rodent animals and Type II from ruminants. Strains isolated from the disease in man fell into one or the other of the types. Julianelle[4] later observed that filtrates from broth cultures gave a precipitate only with their type specific immune sera. The precipitate was suggestive of that seen in the precipitation of specific polysaccharides in the presence of immune serum. The purpose of this paper is to describe the precipitation reactions of 16 strains of *Listeria monocytogenes.*

Material and Methods. The precipitinogen was a polysaccharide extracted from the bacterial cell according to the method of Fuller.[5] Chemically, the carbohydrate fractions were characterized by strongly positive Molisch reactions, and Fehling's solution was not reduced. The biuret and ninhydrin reactions were negative. For the preparation of polysaccharide and the immunization of rabbits bacteria in the smooth phase were selected. All cultures were grown in tryptic digest broth containing 0.1% dextrose and incubated 18 hours at room temperature (25-30° C).

Vaccines were prepared from broth cultures, which were centrifuged and the bacterial sediment resuspended in normal salt solution containing 0.2% formalin. The vaccine was injected intravenously into adult rabbits until the precipitin titer of the serum was sufficient to give a marked, compact-disc precipitate in the presence of the specific carbohydrate within a period of 15 minutes at room temperature. The animals were then bled to death from the heart. Two rabbits were immunized against each strain and the sera from the final bleedings pooled. Control sera were taken from all rabbits before the administration of vaccine and tested for the presence of precipitins to the carbohydrate of that strain of organism to be used for the production of immune serum. In no instance were normal precipitins demonstrated.

Precipitation as determined by the ring test using equal amounts of carbohydrate and immune serum was indicative; however, it

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[1] Paterson, J. S., *J. Path. and Bact.*, 1940, **51**, 424.

[2] Schultz, E. W., Terry, M. C., Brice, A. T., Jr., and Gebhardt, L. P., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 605.

[3] Julianelle, L. A., and Pons, C. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 364.

[4] Julianelle, L. A., *J. Bact.*, 1941, **42**, 367.

[5] Fuller, A. T., *Brit. J. Exp. Path.*, 1938, **19**, 130.

TABLE I.

Precipitation of Carbohydrate Derived from *Listeria monocytogenes* in Antisera of Different Strains.

Antisera against strains of *L. monocytogenes*	Source of strain	Carbohydrates from strain											
		Strain X			Murray No. 204			R-1			Gibson		
		0.4	0.1 cc	0.02	0.4	0.1 cc	0.02	0.4	0.1 cc	0.02	0.4	0.1 cc	0.02
Strain X	Unknown	+	+++	+++	—	++++	++++	—	+++	++++	—	+++	++++
R-1	Rabbit	++	+++	++++	+	++++	++++	++	++++	++++	+	++++	++++
Gibson	Man	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Nyfeldt	"	+	++++	+++	++	++++	+++	+	++++	+++	++	++++	++++
Julianelle	"	++	++++	++++	++	++++	++++	++	++++	+++	+	++++	+++
Schultz	"	—	++++	+++	+	+	++++	—	++	+++	—	++	+++
Murray No. 204	Rabbit	+++	++++	++	++	++++	+++	++	++++	+++	+	+++	++++
Bovine 7647	Cow	—	—	—	—	—	—	—	—	—	—	—	—
S-1	Sheep	+	—	—	++	—	—	+	—	—	+++	++	+
H-14	Man	—	—	—	++	—	—	—	+	—	++	+	—
B-8	Cow	—	—	—	—	—	—	—	—	—	—	—	—
D-82-N	Cattle	—	—	—	—	—	—	—	—	—	++	—	—
G-1	Goat	—	—	—	—	—	—	—	—	—	—	—	—
Burn	Man	—	—	—	—	—	—	—	—	—	—	—	—
Seastone	Fowl	—	—	—	—	—	—	—	—	—	—	—	—

Antisera against strains of *L. monocytogenes*	Source of strain	Carbohydrates from strain											
		Nyfeldt			Julianelle			Schultz			Bovine 7647		
		0.4	0.1 cc	0.02	0.4	0.1 cc	0.02	0.4	0.1 cc	0.02	0.4	0.1 cc	0.02
Strain X	Unknown	—	—	—	—	++	—	—	++	+++	—	—	—
R-1	Rabbit	—	—	—	+	++++	+++	+	++++	++++	—	—	—
Gibson	Man	—	+	+	++	++++	+++	++	++++	+++	—	—	—
Nyfeldt	"	++++	++++	++++	+	++++	+++	+	++++	++++	—	—	—
Julianelle	"	—	—	—	++	++++	++++	+	++++	++++	—	—	—
Schultz	"	—	—	—	++++	+	—	+++	++	—	—	—	—
Murray No. 204	Rabbit	—	—	—	++++	++	—	+++	++++	+	—	—	—
Bovine 7647	Cow	—	—	—	—	—	—	—	—	—	++	++++	++++
S-1	Sheep	+	—	—	++	+	—	+	—	—	++++	++++	++++
H-14	Man	++	+	—	+	++	—	+	++	—	+++	+++	+++
B-8	Cow	—	—	—	—	—	—	—	—	—	—	++++	++++
D-82-N	Cattle	—	—	—	+	—	—	+	—	—	+	+++	+++
G-1	Goat	—	—	—	—	—	—	—	—	—	+	+++	++++
Burn	Man	—	—	—	—	—	—	—	—	—	+++	++++	++++
Seastone	Fowl	—	—	—	—	—	—	—	—	—	—	—	+

TABLE I (Cont.)

Antisera against strains of *L. monocytogenes*	Source of strain	Carbohydrates from strain											
		B-8			S-1			G-1			H-14		
		0.4	0.1 cc	0.02	0.4	0.1 cc	0.02	0.4	0.1 cc	0.02	0.4	0.1 cc	0.02
Strain X	Unknown	—	—		—	—	—	—	—	—	—	—	—
R-1	Rabbit	—	—	—	—	—		—	—	—	—	—	—
Gibson	Man	—	—	—	—	—	—	—	—	—	—	—	—
Nyfeldt	,,	—	—	—	—	—	—	—	—	—	—	—	—
Julianelle	,,	—	—	—	—	—	—	—	—	—	—	—	—
Schultz	,,	—	—	—	—	—	—	—	—	—	—	—	—
Murray No. 204	Rabbit		—		—	—	—	—	—	—	—	—	—
Bovine 7647	Cow	—	—	—	++	++++	++++	+++	++++	++++	++	++++	++++
S-1	Sheep	++++	++	—	++++	++++	++++	++++	+++	++	+++	+++	++
H-14	Man	++	+	—	++	++++	+++	++++	++++	++	++	++++	+++
B-8	Cow	—	+++	—	—	+++	++++	—	++++	++	—	++++	+++
D-82-N	Cattle	++	—	—	+	+++	+++	+++	+++	++	—	+++	+++
G-1	Goat	—	+	++	+	+++	++++	+	+++	++++	+	+++	++++
Burn	Man	—	—	—	++	++++	++++	++++	++++	++++	++	++++	++++
Seastone	Fowl	++	+++	+	—	—	++	—	++	+	—	+	++

Antisera against strains of *L. monocytogenes*	Source of strain	Carbohydrates from strain											
		Burn			D-82-N			Virginia			Seastone		
		0.4	0.1 cc	0.02	0.4	0.1 cc	0.02	0.4	0.1 cc	0.02	0.4	0.1 cc	0.02
Strain X	Unknown	—	—	—	—	—	—	—	—	—	—	—	—
R-1	Rabbit	—	—	—	—	—	—	—	—	—	—	—	—
Gibson	Man	—	—	—	—	—	—	—	—	—	—	—	—
Nyfeldt	,,	—	—	—	—	—	—	—	—	—	—	—	—
Julianelle	,,	—	—	—	—	—	—	—	—	—	—	—	—
Schultz	,,	—	—	—	—	—	—	—	—	—	—	—	—
Murray No. 204	Rabbit	—	—	—	—	—	—	—	—	—	—	—	—
Bovine 7647	Cow	+	+	+	++	++++	++++	—	++++	+	++	++	+
S-1	Sheep	++	+++	+++	+++	+++	+++	++	++++	++++	+	+++	++++
H-14	Man	++	++++	+++	+	++++	++++	+	++++	++++	+	++	++
B-8	Cow	—	+++	++	—	++++	++++	+	+++	++++	—	++++	++++
D-82-N	Cattle	+	+++	+++	+	++	+++	+	++++	+++	+	+	+
G-1	Goat	—	—	—	+	+++	++++	+	+++	++++	—	+	++
Burn	Man	+++	++++	++++	+++	++++	++++	+	++++	++++	—	—	—
Seastone	Fowl	—	—	—	—	+	+	—	—	—	—	+	++++

— = No visible precipitate; + to ++++ = Various degrees of visible precipitation.

seemed advisable in this case to use tubes containing 3 dilutions of the specific soluble substance. Subsequently, precipitation tests were carried out in 10mm x 100mm tubes using 0.4 cc, 0.1 cc, and 0.02 cc amounts of specific soluble substance for each strain of *L. monocytogenes*. Sufficient normal salt solution was added to the tubes containing 0.1 cc and 0.02 cc to bring the final volume in the tube to 0.4 cc. The carbohydrate dilution in each tube was then layered with 0.2 cc of immune serum and the tubes allowed to stand at room temperature for 30 minutes. At the end of this time the presence or absence of ring formation was noted. The tubes were shaken gently and then incubated for 2 hours in a water bath at 37° C. Final readings were taken after storage in the refrigerator overnight at 10° C. and recorded as +, ++, +++ and ++++. In the case of the +++ and ++++ reactions the ring tests were definite at the end of 30 minutes, and usually the precipitate was visible within 10 minutes after adding the serum.

Results. Table I gives the results of the precipitation reactions among 16 strains of *Listeria monocytogenes* studied. On the basis of the precipitation of the carbohydrates in antibacterial sera the strains fell into 2 rather distinct immunological groups, a rodent group and a ruminant group. The existence of 2 immunological groups has been suggested previously by the agglutination reactions. Of the strains used in this study the "Virginia" is possibly the most recent, having been isolated in 1944 from the brain at necropsy of a case of encephalitis occurring in a cow. It was readily identified with the ruminant group by means of the precipitin test. From the table it may be seen that the antibacterial sera of strains S-1 and H-14 show some cross precipitation with the carbohydrates of the rodent group, but the reactions do not appear marked and are not regarded as significant. Antibacterial serum against the Nyfeldt strain precipitated the carbohydrates of the rodent group; however, the Nyfeldt carbohydrate, although reacting with its homologous immune serum, failed to precipitate with the antibacterial sera prepared against the rodent strains. This phenomenon remained unexplained. Polysaccharides prepared from 8 unrelated species of bacteria failed, in all but two instances, to precipitate with antibacterial sera of *Listeria monocytogenes*. Nyfeldt and Gibson antisera precipitated the carbohydrate of hemolytic *Staphylococcus aureus*. The carbohydrates of *Listeria monocytogenes* did not precipitate in antistreptococcal, antityphoid, and antidysentery (Flexner) sera. It is of interest that among the strains from human infections a few are identified as belonging to the rodent group while others fall into the ruminant group.

Discussion. The origin of human infections has remained an enigma; however, it seems entirely possible that they may be derived from some animal source. Carey[6] considered this possibility in dealing with a case of acute cerebrospinal meningitis caused by *L. monocytogenes*. As far as could be determined, in this particular case, the child had had no known contact with rabbits or other animals. It is interesting to speculate that further classification of human infection might follow if the specific soluble substance were to be demonstrated in the cerebrospinal fluid from cases of *Listeria meningitis* and encephalitis. Direct precipitation of the soluble substance from the spinal fluid in the presence of specific immune serum might readily occur. It is suggested that such a procedure if established would possibly prove to be of diagnostic value.

Summary. Polysaccharides have been isolated from *Listeria monocytogenes* which precipitate in the presence of their specific immune sera. The immunological studies presented indicate the existence of 2 distinct animal groups, namely a rodent group and a ruminant group, thus corroborating the classification suggested by Schultz[2] and Julianelle.[3]. The precipitin test affords a simplified technic for the identification and immunological differentiation of the members of the genus Listeria.

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[6] Carey, B. W., Jr., *J. Pediat.*, 1936, **8**, 626.

I wish to thank Drs. E. W. Schultz and M. L. Robbins for the strains of *L. monocytogenes* which they so kindly made available. Also Dr. R. D. Hatch for the strain which was isolated in Virginia in 1944 and which for convenience has been referred to as the Virginia strain.

15219

Changes in Weight of Muscles of Arthritic and Immobilized Arthritic Joints.*

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We have previously studied the synaptic mechanism in muscles of inflamed joints (Frugone, Thomsen, and Luco[1]). In view of the results reported by Thomsen and Luco,[2] we thought it would be interesting to study the changes in weight of such muscles, and the possibility of modifying the weight through immobilization in different positions.

Methods. Cats were used. Immobilization was carried out under ether or sodium pentobarbital (Nembutal Abbott) anesthesia, using bandages impregnated with plaster directly on the skin, extending from the metatarsal region to just below the knee. The arthritis was produced by injecting 0.1 cc of a 5-10% solution of silver nitrate into the articular cavity of the tibia-tarsal joint. The cast was applied immediately after the injection of the silver nitrate. We immobilized in the extreme positions of extension and flexion, that is, in positions of forced hyperextension and hyperflexion held by the cast. The soleus and tibialis anticus muscles were weighed 2.5 to 16 days later, immediately after extirpation. As some of the animals developed marked edema of the immobilized extremity, we ran

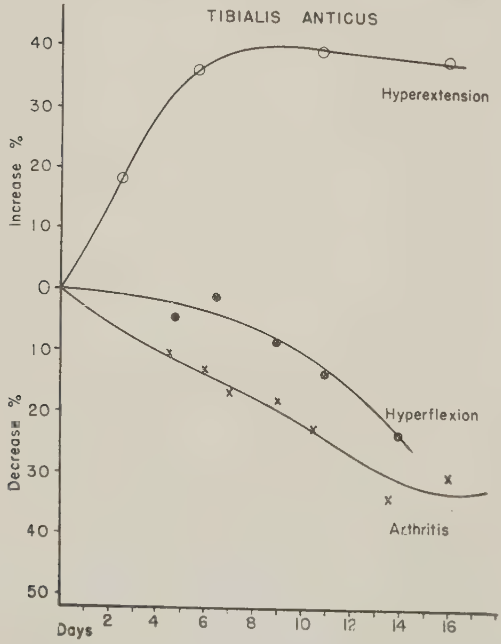

Fig. 1.
Changes of weight of the tibialis anticus in arthritis, arthritis with immobilization in hyperflexion, and arthritis with immobilization in hyperextension. The curves represent the plotted average values of Tables I, III, and V respectively.

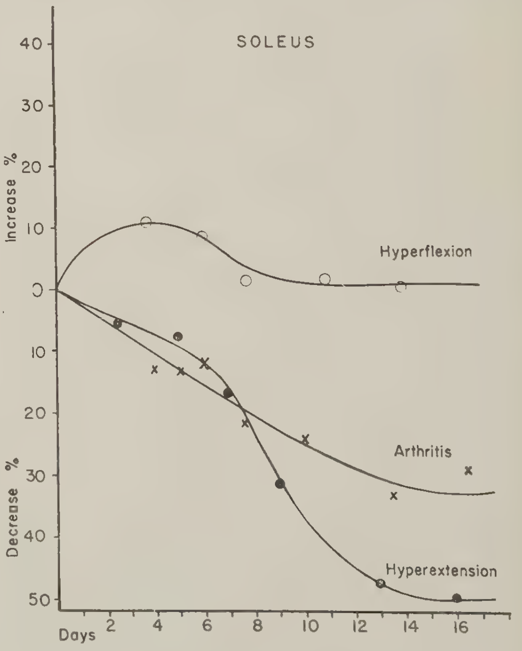

Fig. 2.
Changes of weight of the soleus in arthritis, arthritis with immobilization in hyperflexion, and arthritis with immobilization in hyperextension. The curves represent the plotted average values of Tables II, IV, and VI respectively.

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* Aided by a grant from the National Foundation for Infantile Paralysis.

[1] Frugone, J. E., Thomsen, P., and Luco, J. V., in press.

[2] Thomsen, P., and Luco, J. V., *J. Neurophysiol.*, 1944, **7**, 245.

TABLE I.
Showing Loss of Weight of Tibialis Muscle in Arthritis.

Animal No.	Days of arthritis	Wt in g		% difference
		N	A	
1	4	4,930	4,620	—6.5
2	4	2,600	2,300	—11.
3	4	3,200	3,120	—2.5
4	5	750	560	—25.3
5	5	4,975	4,625	—7.
	Avg 4.4			—10.4 ± 3.1
6	6	3,400	2,800	—17.
7	6	2,620	2,370	—9.
	” 6			—13. ± 4.
8	7	3,470	3,170	—8.
9	7	3,080	2,435	—19.
10	7	4,620	3,550	—23.
	” 7			—16.6 ± 4.46
11	8	4,690	3,980	—14.
12	8	2,700	2,200	—18.
13	8	2,910	2,350	—19.2
14	8	3,060	2,570	—16.
	” 8			—16.8 ± 0.37
15	9	4,320	3,600	—15.
16	9	1,380	1,130	—18.1
17	9	2,080	1,770	—14.9
	” 9			—16. ± 1.
18	10	3,750	2,520	—32.
19	10	2,970	2,570	—13.
20	10	880	810	—8.
21	10	3,600	2,750	—23.
22	11	3,700	2,550	—31.
23	11	3,190	2,200	—31.
24	11	4,190	3,280	—21.
	” 10.4			—22.7 ± 3.58
25	13	2,220	1,330	—40.
26	13	4,310	4,070	—5.5
27	14	3,100	1,780	—43.
28	14	4,500	2,340	—48.
	” 13.5			—34.1 ± 9.55
29	16	4,600	3,800	—17.
30	17	2,640	1,470	—44.
	” 16.5			—30.5 ± 13.52

N—Normal.
A—Arthritis.

several controls with muscles desiccated in the oven, at 105°C for 24 hours, to eliminate the excess of water.

Results. A. Weight of Muscles in Arthritis. The results of the 30 experiments in this series given in Tables I and II, show that the percentage loss of weight of the tibialis anticus and the soleus is similar and varies with the

TABLE II.
Showing Loss of Weight in Soleus Muscle in Arthritis.

		Wt in g		
Animal No.	Days of arthritis	N	A	% difference
1	4	2,970	1,800	—10.
2	4	1,450	1,200	—17.
3	4	1,850	1,620	—12.
	Avg 4			—13. ± 2.
4	5	670	560	—16.4
5	5	2,375	2,120	—10.
	" 5			—13.2 ± 3.
6	6	2,100	1,900	— 9.5
7	6	2,240	1,900	—15.
	" 6			—12.3 ± 2.45
8	7	1,885	1,875	— 5.5
9	7	1,970	1,520	—22.
10	7	2,480	1,820	—26.
11	8	2,550	2,200	—13.
12	8	2,000	1,800	—10.
13	8	2,680	1,520	—43.2
14	8	1,730	1,120	—35.2
	" 7.6			—21.4 ± 5.55
15	9	2,300	1,930	—16.
16	9	1,040	680	—34.6
17	9	1,510	920	—39.
18	10	1,610	1,190	—26.
19	10	2,580	2,050	—21.
20	10	700	510	—27.
21	10	1,900	1,700	—11.
22	11	1,950	1,600	—19.
23	11	2,240	1,530	—32.
24	11	2,170	1,930	—15.
	" 10			—24. + 2.91
25	13	1,350	1,050	—22.
26	13	2,720	2,010	—26.1
27	14	1,880	1,160	—38.
28	14	1,820	1,320	—27.4
	" 13.5			—33.3 ± 4.35
29	16	2,930	2,420	—17.
30	17	2,070	1,300	—37.
	" 16.5			—27. ± 10.

N—Normal.
A—Arthritis.

number of days of arthritis. This relationship is seen more clearly in Fig. 1 and 2.

B. *Weight of Muscles of Arthritic Joints Immobilized in Hyperflexion.* In this series we used 28 animals and the results are shown in Tables III and IV, and Figs. 1 and 2. In this position, the soleus after an initial increase tends to maintain its normal weight. The tibialis anticus undergoes atrophy, but not as much as in the case of pure arthritis, although the weight of desiccated muscles shows that the loss of weight is similar to that produced by the arthritis alone.

C. *Weight of Muscles of Arthritic Joints*

TABLE III.
Tibialis in Arthritis with Hyperflexion.

Animal No.	Days of arthritis	Wt in g N	Wt in g AI	% difference	Wt of desic. muscles N	Wt of desic. muscles AI	% difference
61	2.5	1,950	1,890	— 3			
62	2.5	3,440	3,460	+ 0.5	940	890	— 5.3
63	2.5	2,100	2,150	+ 2.3	560	560	0
64	2.5	2,470	2,480	+ 0.4	685	700	— 2.1
65	2.5	1,150	1,080	— 6	350	325	— 7.1
Avg	2.5			— 1.1 ± 1.15			
66	4	3,470	3,370	— 2.8			
67	5	3,410	3,320	— 2.6			
68	5	3,290	3,310	+ 0.6	860	850	— 1.1
69	5	4,970	4,320	—13.1			
70	5	4,460	4,000	—10.1			
71	5	3,300	3,300	0			
”	4.8			— 4.6 ± 2.22			
72	6	4,820	4,930	+ 2.7			
73	6	2,270	2,080	— 8.4			
74	6	5,470	4,760	—14.8	1,150	1,120	— 2.6
75	6	4,120	4,280	+ 3.8	1,130	1,160	+ 2.6
76	6	5.550	5,570	+ 0.3	1,430	1,420	— 0.7
77	7	3,370	2,870	—15			
78	7	2,376	2,570	+11.9			
79	7	2,030	2,210	+ 8.7			
80	8	3,530	3,550	+ 0.5	1,040	940	— 9.6
”	6.5			— 1.3 ± 3			
81	9	6,000	5,600	— 6.6			
82	9	1,970	1,780	—10			
”	9			— 8.3 ± 1.58			
83	11	3,350	3,600	+ 6.9			
84	11	3,870	2,840	—26.8			
85	11	3,900	3,000	—23			
86	11	2,950	2,640	—10	700	610	—12.8
”	11			—13.7 ± 7.61			
87	14	3,660	3,200	—12.2	990	770	—22.2
88	14	3,520	2,170	—35.3	950	586	—49.9
”	14			—23.7 ± 11.6			

N—Normal.
AI—Arthritis with immobilization in hyperflexion.

Immobilized in Hyperextension. The results in the 30 animals studied are shown in Tables V and VI, and Fig. 1 and 2. The soleus undergoes more atrophy than in the case of pure arthritis. The tibialis anticus shows an increase in weight that is evident both in the fresh and desiccated muscles. The figures for desiccated muscles are lower but tend to parallel those for fresh muscles.

Discussion. The loss of weight caused in the soleus and the tibialis anticus by an arthritis of the tibio-tarsal joint is very similar to that described by Lippman and Selig.[3] The curve obtained by those authors in a series of 40 animals, by plotting the loss of weight against the duration (in days) of arthritis, practically coincides with the curve that we

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[3] Lippman, R. K., and Seliz, S., *Surg. Gyn. Obst.*, 1928, **47**, 512.

TABLE IV.
Soleus in Arthritis with Hyperflexion.

Animal No.	Days of arthritis	Wt in g N	Wt in g AI	% difference	Wt of desic. muscles N	Wt of desic. muscles AI	% difference
61	2.5	1,210	1,530	+26.2			
62	2.5	1,730	1,950	+11.2	450	480	+ 6.2
63	2.5	1,180	1,220	+ 3.4	310	330	+ 6.4
64	2.5	1,060	1,220	+15	300	345	+15
65	2.5	750	1,000	+31.8	220	280	+27.2
66	4	2,440	2,340	— 4.1			
67	5	2,370	2,460	+ 3.6			
68	5	2,430	2,360	— 2.8	540	560	+ 3.7
69	5	2,200	2,770	+25.8			
70	5	2,620	2,740	+ 4.6			
71	5	1,830	1,890	+ 3.2			
Avg.	3.7			+10.7 ± 3.74			
72	6	2,700	3,000	+11			
73	6	1,330	1,470	+10.5			
74	6	2,250	2,580	+14.6	520	600	+15.3
75	6	3,000	2,930	— 2.3	770	770	0
76	6	2,480	2,690	+ 8.4	630	640	+ 1.6
”	6			+ 8.4 ± 2.82			
77	7	2,030	1,450	—28.5			
78	7	1,050	1,080	+ 2.8			
79	7	1,530	2,018	+32			
80	8	2,670	2,400	—10.1	670	610	— 8.9
81	9	3,650	3,450	— 5.4			
82	9	1,370	1,620	+18.3			
”	7.8			+ 1.45 ± 8.78			
83	11	1,850	2,220	+15.8			
84	11	2,220	2,370	+ 6.4			
85	11	2,550	2,410	— 5.5			
86	11	1,590	1,400	—11.9	380	360	— 5.2
”	11			+ 1.7 ± 5.66			
87	14	1,870	1,850	— 1	450	450	0
88	14	2,550	2,600	+ 1.9	630	650	+ 3.1
”	14			+ 0.4 ± 1.58			

N—Normal.
AI—Arthritis with immobilization in hyperflexion.

obtained for the tibialis in a series of 30 animals.

The muscular atrophy produced by arthritis is different from that obtained by immobilization and as observed by Thomsen and Luco; in the former case both muscles undergo atrophy, while in the latter case only the muscle that is in a state of maximum shortening undergoes atrophy.

If we combine these two conditions that cause changes of weight, *i.e.*, arthritis and immobilization in an extreme position, we observe that in some cases we obtain a summation while in others we do not. In Table VII, we show the average percentage of change in weight in the group of animals studied after 10-11 days of treatment, together with the results obtained by Thomsen and Luco[2] for the same period.

Table VII shows that the loss of weight of the soleus in arthritis is slightly over one-half the loss of weight in immobilization in hyperextension, and that the loss of weight in arthritis with hyperextension is practically

TABLE V.
Tibialis in Arthritis with Hyperextension.

Animal No.	Days of arthritis	Wt in g		% difference	Wt of desic. muscles		% difference
		N	AI		N	AI	
31	2.5	3,600	4,220	+17.2	1,080	1,170	+ 8.3
32	2.5	2,630	3,600	+36.8	780	870	+11.5
33	2.5	3,280	3,440	+ 4.8	920	980	+ 6.5
34	2.5	3,300	3,920	+18.8	820	1,020	+24.3
35	2.5	1,710	2,040	+13.4			
	Avg 2.5			+18.2 ± 5.2			
36	5	5,750	6,500	+13			
37	5	3,020	3,990	+32.1			
38	5	2,900	3,960	+40			
39	5	3,170	4,640	+46.3	770	1,100	+42.8
40	5	4,480	7,270	+62.2	1,130	1,410	+24.7
41	5	3,560	5,000	+40.4	870	1,050	+17.1
42	7	4,500	6,220	+28			
43	7	3,280	4,320	+24			
44	7	3,180	4,380	+37.7	830	1,050	+26.5
	” 5.7			+35.9 ± 4.68			
45	9	5,200	6,250	+17			
46	9	4,280	5,560	+22.8	1,110	1,220	+ 9.9
47	9	3,250	3,820	+17.5	830	960	+13.5
48	9	3,420	4,610	+25.8	840	1,120	+33.3
49	9	2,250	3,130	+39.1			
50	10	3,940	5,300	+26			
51	12	3,780	5,460	+17.9	1,020	1,220	+19.6
52	13	2,360	4,420	+87.2	590	970	+64.4
53	13	4,200	4,040	— 3.8			
54	13	1,330	2,610	+96.2	380	640	+68.4
55	14	1,510	2,760	+82.8	440	700	+59
	” 10.8			+38.9 ± 10			
56	16	3,900	5,600	+43.6	1,050	1,390	+32.3
57	16	2,830	3,920	+38.5	790	1,060	+34.1
58	16	2,720	3,170	+15.2			
59	16	3,640	5,980	+64.5	1,000	1,190	+19
60	16	4,610	6,430	+25.3	1,280	1,620	+26.7
	” 16			+37.2 ± 8.6			

N—Normal.
AI—Arthritis with immobilization in hyperextension.

the same as that with hyperextension alone.

Hyperflexion results in a slight increase in weight of the soleus, and arthritis plus hyperflexion results in no change of weight, the hyperflexion preventing the loss of weight that usually accompanies arthritis.

In the case of the tibialis anticus, in which arthritis causes a loss of and hyperextension an increase of weight, the simultaneous action of these two factors results in an increase of weight greater than by immobilization alone. No doubt edema plays a role in these results. Also, the standard error of the increase in weight is ±10, indicating that the mean value may be subject to great variation.

Hyperflexion causes a loss of weight of the tibialis, but when combined with arthritis it fails to alter the loss of weight due to the latter.

Summarizing: the loss of weight produced by immobilization and shortening of a muscle is not modified by arthritis in a period of 10 or 11 days. On the other hand, the atrophy due to arthritis does not develop, or is changed into hypertrophy when the joint is immobilized in a position which by itself causes an increase in weight.

As we said before, the increase in weight of the tibialis by immobilization and arthritis

TABLE VI.
Soleus in Arthritis with Hyperextension

Animal No.	Days of arthritis	Wt in g N	Wt in g AI	% difference	Wt of desic. muscles N	Wt of desic. muscles AI	% difference
31	2.5	2,080	1,850	—11.1	540	510	— 5.5
32	2.5	1,730	1,740	+ 0.5	450	450	0
33	2.5	1,250	1,360	+ 8.8	360	380	+ 5.5
34	2.5	2,330	2,070	—11.1	630	560	—11.1
35	2.5	1,370	1,200	—14.2			
Avg	2.5			— 5.4 ± 4.32			
36	5	2,850	2,800	— 2			
37	5	1,740	1,630	— 6.3			
38	5	1,950	1,710	—14			
39	5	1,800	1,230	—31.6	420	320	—23.8
40	5	1,740	1,680	— 3.4	440	390	—11.3
41	5	1,480	1,640	—10.6	420	430	+ 2.3
”	5			— 7.7 ± 5.63			
42	7	3,100	3,000	— 3			
43	7	1,600	1,400	—12			
44	7	2,300	1,490	—35.2	520	370	—28.8
”	7			—16.7 ± 9.5			
45	9	2,550	1,830	—28			
46	9	2,080	1,350	—35.5	450	330	—26.6
47	9	1,700	890	—47.6	470	290	—38.3
48	9	1,950	1,340	—31.2	470	310	—34
49	9	1,320	1,040	—21.2			
50	10	1,970	1,430	—27			
”	9.1			—31.6 ± 3.16			
51	12	1,690	1,130	—33.1	460	280	—39.1
52	13	1,740	900	—48.2	440	220	—50
53	13	1,820	940	—48.4			
54	13	1,060	440	—58.5	275	110	—60
55	13	1,160	600	—48.3	310	170	—45.1
”	12.8			—47.3 ± 4.15			
56	16	2,640	1,330	—45.4	670	330	—50.7
57	16	1,730	660	—62	430	190	—55.8
58	16	1,960	610	—69			
59	16	2,090	1,510	—27	550	290	—47.2
60	16	2,270	1,290	—43.7	620	360	—41.8
”	16			—49.4 ± 7.36			

N—Normal.
AI—Arthritis with immobilization in hyperextension.

is less marked in desiccated muscles. This applies to both positions. On the other hand, the results obtained with desiccated soleus muscle are similar to those obtained with the fresh muscle.

With reference to periarthritis, Lippman and Selig discarded all animals that developed this complication and, nevertheless, obtained a curve that almost coincides with the one we obtained without discarding such animals.

The results obtained in this work do not agree with those of Huddleston,[4] who found that immobilization of the tibialis in a position

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[4] Huddleston, O. L., *South, M. J.*, 1944, **37**, 72.

TABLE VII.

	% diff. in soleus	% diff. in tibialis
Arthritis	—24.0	—22.7
Arthritis and hyperextension	—38.8	+38.9
Hyperextension	—38.0	+28.0
Arthritis and hyperflexion	+ 2.9	—11.6
Hyperflexion	+11.0	—13.0

of maximum extension (hyperextension) caused a greater loss of weight than immobilization in maximum shortening, and that the change in the soleus was the same in either position. The experimental conditions were different in the two cases, and we believe that the different results are due to the fact that we studied the first 16 days of immobilization while Huddleston's experiments extended from 95 to 290 days. It may be that our results would change with time and come to resemble those of Huddleston. Besides, the results listed in our tables show that there are wide variations from animal to animal and, therefore, the averages must be taken from a large number of animals.

Our results cannot be compared either with those of Hines[5] who studied in rats the effect of immobilization on the recovery from paralysis caused by crushing the nerve. In our experiments the nerve was kept intact.

Summary. 1. Arthritis of the tibio-tarsal joint produced in cats by injecting silver nitrate, causes an atrophy of the soleus and tibialis anticus muscles. Tables I and II, Fig. 1 and 2.

2. Immobilization of the inflamed tibio-tarsal joint in hyperflexion results in a loss of weight of the tibialis that is similar or slightly less marked than that caused by arthritis alone. Table III, Fig. 1. In these conditions the soleus keeps its normal weight. Table IV, Fig. 2.

3. Immobilization of the inflamed joint in hyperextension results in an increase of weight of the tibialis. Table V, Fig. 1. The soleus, on the other hand, shows a greater loss of weight than by arthritis alone. Table VI, Fig. 2.

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[5] Hines, H. M., *J. A. M. A.*, 1942, **120**, 515.

15220

Respiratory Mask and Valves for Dogs.

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The units to be described were developed in this laboratory for the purpose of collecting expired air of unanesthetized, trained dogs. The avoidance of leaks, minimization of resistance to breathing and of dead space were prime factors considered in designing the apparatus. No new fundamental principles are involved; rather, the apparatus embodies good features of older designs, eliminates some of the bad and utilizes new devices, to our knowledge never previously described.

Mask. The mask consists of a copper wire frame, on the order of carefully shaped commercial wire muzzle, made to fit loosely the snout of a 15 kg mongrel hound. The nasal end is projected about 1″ beyond the nose and fitted with a No. 9 stopper with a single ⅜″ bore. The open end is bent to fit the snout near the lip corners with about ¼″

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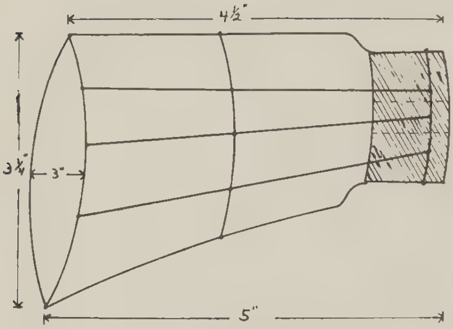

FIG. 1
Mask frame without covering. Shaded portion a No. 9 rubber stopper for insertion of B in Fig. 2.

clearance. Eight longitudinal wires spaced evenly around the frame serve to give good support and shape to the covering material. The finished frame (Fig. 1) is wrapped with 3 or 4 thicknesses of plaster bandage, allowed to dry overnight and soaked for an hour in melted paraffin. A latex surgeon's glove, size 8, from which the fingers are removed is stretched over the open end of the mask and secured by wrapping tightly with string and brushing with molten paraffin; about 3″ of the wrist end of the glove is allowed to project as a sleeve. In use, the sleeve is stretched over the dog's muzzle to beyond the lip corners and invaginated into the mask by sliding the latter toward the eyes. Two rubber strips cemented to the end of the sleeve and tied together behind the ears prevent rolling of the glove and exposure of the lip corners during a measurement. We have found no grease to be necessary to prevent leaks under the glove; this may, however, be necessary in the case of very long-haired animals. The mask can be steadied during the experiment either by hand or a clamp. Such a mask will fit a number of 10-20 kg animals with similar muzzle shapes thus obviating the necessity of individually molded outfits. For smaller animals a smaller size glove may be used as sleeve, but the mask dead-space factor may become significant in such cases. Hence we have constructed similar but smaller size masks which may be used for 5-10 kg dogs. Although this mask does not fit the muzzle tightly enough to prevent some opening of the jaws it would limit the extent of such movement and thus might limit the efficiency and degree of the panting process.

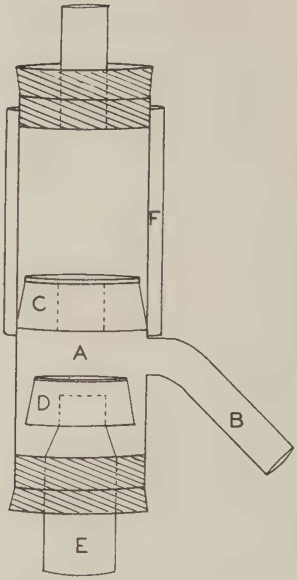

FIG. 2
Respiratory valves, shown in position used.

Valves. The valves are of the ordinary rubber flap type enclosed in glass. (A) in Fig. 2 is a 5¼″ long, 45 mm o.d. standard Pyrex tube with a 13 mm o.d. tube (B) fused to one side about ⅓ of the distance from one end. The expiratory valve (C) is a No. 8 rubber stopper with ⅝″ bore covered with a circle of 1/32″ rubber sheet secured to the stopper by a small dot of rubber cement on one edge. A perfectly flat piece of sheet rubber and a smooth stopper must be selected to avoid leaks. The inspiratory valve (D) is a No. 7 stopper covered with a similar flap. The 25 mm o.d. glass tube (E) should produce a minimum of stretching of the stopper (D) when inserted to prevent distortion of the valve seat and consequent leaks. The ends of (A) are closed with single bore No. 9 stoppers which can be connected to spirometers. A 20-watt electric heater (F) is built to fit over the upper half of the valve. This prevents condensation of moisture on the expiratory valve during collection and eliminates the objectionable sticking of this flap with its consequent resistance to expiration. The valve should be used only in or very close to the vertical position as shown in the diagram as otherwise the flaps will be opened by gravity between respirations allowing significant leaks. Small springs to close the flaps might eliminate this danger. The valve should be stored in the vertical position when not in use. The tube (B) with a 45° bend, inserted into the stopper of the mask, is used when the dog is supine with head tilted 45° upward. Modification of this bend permits use with the animal in any desired position. Modification of the diameter and bend of (B) and attachment to a suitable mask permits use on man. No significant resistance to respiration develops with these valves even during exercise or CO_2 inhalation.

15221

Effect of Salicylate on Plasma Fibrinogen and Sedimentation Rate in Rheumatic and Non-Rheumatic Patients.*

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Despite the long-standing use of salicylates in the management of rheumatic fever, considerable uncertainty still exists with regard to their efficacy and to their mode of action in this disease. While some observers believe that their clinical effect may be ascribed entirely to the analgesic and antipyretic action,[1] others have assumed a specific inflammation-suppressant effect of these drugs in rheumatic fever. Studies which indicated a more rapid return to normal of the sedimentation rate in rheumatic patients receiving large doses of salicylates were interpreted as supporting evidence for this view, under the assumption that the sedimentation rate represented an index of inflammatory activity.[2] A specific influence of the drug on the sedimentation rate apart from its assumed action on the inflammation is also suggested by the observation that the sedimentation rate may increase promptly on discontinuance of salicylate therapy.[3]

Present evidence indicates that the sedimentation rate of the erythrocytes is mainly dependent on the concentration and distribution of the various fractions of the plasma proteins.[4] Fibrinogen exerts the greatest accelerating action. A close relationship between the sedimentation rate and the level of fibrinogen has been demonstrated by several observers in various experimental conditions and diseases, among them rheumatic fever.[5] Fibrinogen is generally believed to be formed solely in the liver.[6] In view of these facts it appears possible that the effect of salicylates on the sedimentation rate of rheumatic patients is due to an inhibition of the formation of fibrinogen by the liver. That salicylates may affect liver function is also suggested by the observation that they may cause significant reduction in the plasma prothrombin in animals and in man.[7] One would not expect such

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* Reported at a conference on Liver Injury held under auspices of the Josiah Macy, Jr., Foundation, New York, September 19, 1944.

[1] Hanzlik, P. J., *Medicine*, 1926, **5**, 197.

[2] Coburn, A. F., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 435.

[3] Lichty, J. A., and Hooker, S. P., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 69.

[4] Fåhraeus, R., *Physiol. Rev.*, 1929, **9**, 241; Westergren, A., Theorell, H., and Widstroem, S., *Z. ges. Exp. Med.*, 1931, **75**, 668; Bendien, W. M., Neuberg, J., and Snapper, I., *Bioch. Z.*, 1932, **247**, 306; Grey, S. J., and Mitchell, E. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 403.

[5] Ernstene, A. C., *Am. J. Med. Sc.*, 1930, **180**, 12; Gilligan, D. R., and Ernstene, A. C., *Am. J. Med. Sc.*, 1934, **187**, 552; Ham, T. H., and Curtis, F. C., *Medicine*, 1938, **17**, 447.

[6] Madden, S. C., and Whipple, G. H., *Physiol. Rev.*, 1940, **20**, 194.

[7] Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, C. F., and Scheel, L. D., *J. Biol. Chem.*,

TABLE I.
Changes in Plasma Fibrinogen and Sedimentation Rate Following Salicylate Therapy.

Case No.	Diagnosis	Age, yrs	Dosage, g/kg/day	Time, relative to medication	Plasma fibrinogen, mg/100 cc	Sedimentation rate uncorrected, mm/min.	Sedimentation rate corrected, mm/min.
1	Nephrosis	11	0	before	951	3.63	1.96
			0.13	2 days	857	3.07	1.80
			0.13	5 ”	676	2.86	1.64
			0.13	10 ”	534	2.35	1.33
			0.13	14 ”	468	2.26	1.42
			0	8 ” after	535	2.65	1.90
			0	20 ” ”	764	3.09	2.00
2	”	7	0	before	924	3.72	2.18
			0.15	16 days	652	3.00	1.88
			0	8 ” after	794	4.22	2.14
3	”	8	0	before	653	2.20	2.00
			0.11	9 days	415	1.70	1.38
			0.11	16 ”	184	1.27	1.02
			0	7 ” after	479	1.55	1.33
4	Chron. staph. osteomyelitis	6	0	before	628	2.81	1.68
			0.09	7 days	360	2.52	1.48
			0	7 ” after	690	3.15	1.83
5	” ” ”	3	0	before	501	1.64	1.04
			0.11	7 days	220	1.06	0.65
			0	14 ” after	598	1.06	0.68
6	Brain abscess	14	0	before	858	4.64	1.77
			0.15	4 days	780	2.49	1.40
			0.15	13 ”	583	1.24	1.18
7	Rheum. fever	2	0	before	843	2.50	1.35
			0.20	7 days	608	1.34	0.72
			0.20	20 ”	376	0.73	0.45
8	” ”	12	0	before	858	2.71	1.60
			0.13	5 days	589	2.26	1.42
			0.13	20 ”	404	0.52	0.28
			0	45 ” after	631	0.97	0.78
9	” ”	5	0	before	622	1.86	1.18
			0.17	7 days	552	0.80	0.36
			0.17	29 ”	388	0.19	0.12
10	” ”	7	0	before	614	2.83	1.74
			0.13	14 days	548	2.19	1.18
			0	12 ” after	673	2.32	1.50
11	Nephrosis	7	0	before	785	6.50	2.10
			0.14	7 days	808	6.74	2.12
			0	7 ” after	940	7.57	2.57
12	Brain abscess	8	0	before	726	1.16	0.68
			0.13	12 days	746	1.34	0.74
13	Rheumatoid arthritis	11	0	before	626	2.41	1.70
				10 days	640	2.81	1.94
				12 ” after	740	4.22	2.44
14	Pericarditis und. etiol.	2	0	before	472	1.26	0.72
			0.18	6 days	506	1.16	0.63
15	Rheum. fever	14	0	before	619	—	—
			0.13	9 days	592	0.80	0.66
			0.13	16 ”	614	1.13	0.80

an effect of the salicylates to be limited to rheumatic patients alone; decreases in the plasma fibrinogen and in the sedimentation rate following salicylate medication should be demonstrable in other patients with elevated sedimentation rates, who do not suffer from rheumatic fever. In the present study parallel determinations of the sedimentation rate and of plasma fibrinogen were carried out before, during, and after courses of salicylate adminis-

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1943, **147**, 463; Rapoport, S., Wing, M., and Guest, G. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 40; Meyer, O. O., and Howard, B., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 234; Shapiro, S., Redish, M. H., and Campbell, H. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 251.

tration in patients suffering from various chronic disorders in which the sedimentation rate is elevated.

Material and Methods. Fifteen patients with elevated sedimentation rates, varying in age from 2 to 14 years, were chosen for this study. Eight displayed some manifestations of rheumatic fever, 4 were suffering from lipoid nephrosis, and 3 from chronic osteomyelitis. The subjects received daily 0.09-0.22 g per kg of body weight of acetyl-salicylic acid combined with an equal amount of sodium bicarbonate for a period of 7 to 29 days. Blood samples for the determination of the sedimentation rate and of the plasma fibrinogen were obtained before, during, and after the period of medication at varying intervals of time, usually 3 to 5 days. Most subjects tolerated the drug well, except for one patient with nephrosis who developed hypoprothrombinemia and hematuria; shortly after discontinuance of salicylate medication the hematuria ceased and the prothrombin returned to normal.

The sedimentation rate was determined by the method of Rourke and Ernstene[8] using heparinized blood with values corrected for the packed volume of cells. Fibrinogen was determined as fibrin, following essentially the procedure of Cullen and Van Slyke.[9]

Results. Ten of the patients, whose data are summarized in Table I, exhibited significant reduction in the fibrinogen level of their plasma during periods of salicylate medication. In all instances the sedimentation rate of the erythrocytes was also reduced, but the extent of the decrease varied considerably. In the other 5 patients, in whom the plasma fibrinogen did not decrease, no important changes were observed in the sedimentation rate. Several striking discrepancies between the two indices, demonstrated particularly in Cases 3, 5, and 8 in Table I, would indicate that other factors besides fibrinogen were operative in determining the sedimentation rate. It well may be that the effect of the salicylates on the distribution of plasma protein, and with it on the sedimentation rate, is not limited to fibrinogen alone, although determination of albumin and globulin by the conventional Howe method in several of the samples did not indicate significant change. The data on Cases 1 to 6 would indicate that salicylates may lower the plasma fibrinogen and the sedimentation rate in conditions unrelated to the rheumatic disorder. Such findings justify the assumption that salicylates exert an unspecific action on the liver, and indirectly support the contention of those observers who believe that salicylates do not have a specific anti-rheumatic action. However, it is of course conceivable that changes in the distribution of plasma protein produced by salicylates may in some manner influence favorably the course of rheumatic inflammation.

Summary. In 10 of 15 patients with elevated sedimentation rates, suffering from nonrheumatic and rheumatic disorders, the sedimentation rate and concentration of fibrinogen decreased following salicylate therapy. It is suggested that the change in the fibrinogen may represent an effect of salicylate on the liver.

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[8] Rourke, M. D., and Ernstene, A. C., *J. Clin. Invest.*, 1930, **8**, 545.

[9] Cullen, G. E., and Van Slyke, D. D., *J. Biol. Chem.*, 1920, **41**, 587.

15222

A Test for Penicillin Sensitivity and Resistance in Staphylococcus.*

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The occurrence of penicillin-fast strains of bacterial species generally susceptible to the action of penicillin has been recorded repeatedly.[1] It has been established that a susceptible strain may acquire penicillin resistance *in vivo* following penicillin treatment, or *in vitro* after growth in penicillin-containing media. The mechanism of acquisition of penicillin fastness *in vitro* by staphylococci was studied by Demerec[2] who found that even very susceptible strains of staphylococci give origin to a few variants resistant to small concentrations of penicillin. The variants in turn, once allowed to grow, give variants of higher resistance. In several steps, but never in one step, it is possible to obtain variants resistant to very high concentrations, up to 250 or more units per ml. This type of resistance is generally permanent. Using the statistical method devised by Luria and Delbruck[3] for the study of bacteriophage resistance, Demerec was able to show that these penicillin-fast variants arise by rare mutations from sensitive organisms, independently of the action of penicillin itself. In all probability the drug merely acts as a selective agent which, by inhibiting the growth of sensitive individuals, allows the mutant individuals of sufficient resistance to grow. Resistance to high concentrations is the result of successive mutations, each contributing further resistance.

The situation is complicated by the occurrence of another type of penicillin resistance in staphylococci.[1] Many of the so-called resistant strains isolated from patients, either before or after treatment with penicillin, seem to owe their ability to withstand the antibiotic to the production of a penicillin inactivator, probably of enzymatic nature (penicillinase[4]). Experiments discussed in this paper show that penicillinase production need not be associated with penicillin resistance of the individual cells.

One important requirement in experimental work and in hospital laboratory routine is the availability of a reliable test for penicillin resistance in staphylococcus. The test should indicate the degree of sensitivity of the bulk of a bacterial population and the presence and proportion in it of cells of higher resistance. For penicillinase-producing strains, the test should indicate the degree of sensitivity of individual cells. Such a test is described in the present paper.

Technic. For the experiments on survival in agar containing penicillin pour plates were used. These were made by introducing the desired amount of penicillin, and then a measured bacterial inoculum, into a tube containing 10 ml of melted nutrient agar (at 45°C), mixing the contents of the tube thoroughly, and plating immediately. With this method, no heat inactivation of penicillin takes place. Results were read after 72 hours of incubation at 37°C.

Penicillin was kept in a refrigerator in

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† The author is indebted to Lt. A. Reiss, Sn.C., Base Hospital Laboratory, Mitchel Field, N.Y., and to Miss B. Johnson, Surgical Bacteriology Laboratory, College of Physicians and Surgeons, Columbia University, for supplying cultures of staphylococci, and to Chas. Pfizer and Co. for generous supplies of penicillin. The technical assistance of Miss Rachel M. Arbogast is gratefully acknowledged.

[1] See Spink, W. W., Hall, W. H., and Ferris, V., *J. A. M. A.*, 1945, **128**, 555.

[2] Demerec, M., *Proc. Nat. Acad. Sc.*, 1945, **31**, 16.

[3] Luria, S. E., and Delbruck, M., *Genetics*, 1943, **28**, 491.

[4] Abraham, E. P., and Chain, E., *Nature*, 1940, **146**, 837.

TABLE I.
Survival of Penicillin sensitive Staphylococci in Agar Containing Penicillin.

Penicillin, units/ml	Survival in agar by colony counts Strain NRRL, bacteria/ml	Strain 02, bacteria/ml	Strain 5399, bacteria/ml
0.00	2.2×10^8	2.4×10^8	2.7×10^8
0.01	2.5×10^6		
0.02	2.5×10^4	5.0×10^3	2.0×10^4
0.03	1.0×10^4	4.5×10^2	9.0×10^2
0.04	4.0×10^3	5×10^1	3.0×10^1
0.06	1.3×10^2	6×10^0	
0.08	3.0×10^1	0	1.0×10^1
0.10	0	0	0

TABLE II.
Survival of Staphylococci Made Resistant to Penicillin.

		Penicillin, units/ml	Survival by colony count, bacteria/ml
a. Strain from a colony grown in presence of	0.06 units/ml	0.00	5×10^8
		0.04	3×10^8
		0.08	5×10^5
		0.12	2×10^4
		0.16	4×10^3
		0.24	2×10^2
		0.32	0
b. *idem*	0.4 ”	0.0	7×10^8
		0.2	9×10^6
		0.3	3×10^5
		0.4	1×10^5
		0.5	2×10^3
		0.75	2×10^2
		1.00	0
c. *idem*	1.0 ”	0.0	6×10^8
		1.0	4×10^4
		2.0	1×10^4
		4.0	1×10^3
		6.0	2×10^1
		8.0	1×10^1
		12.0	0

stock solutions of 10,000 units per ml in phosphate buffer of pH 6.9. The stock was titrated at frequent intervals by the cup method,[5] and compared with a penicillin standard. A stock was discarded as soon as its titer began to diminish appreciably.

Tests for penicillinase activity were made by mixing 1 ml of the sample to be tested with 1 ml of a penicillin solution of the desired concentration (generally 40 units/ml). The mixtures were incubated at 37°C for 1 or 2 hours, then assayed, directly or after dilution, by the cup method. Acetone-ether dried preparations for penicillinase tests were made according to Harper.[6]

Results. When sensitive strains of staphylococci are plated in agar containing penicillin, the results are of the type illustrated in Table I.

If bacteria from any of the colonies grown in the presence of the largest amount of penicillin in the experiments of Table I are picked out, grown in broth, and retested in penicillin agar, results of the type illustrated in Table II, a, are obtained. By sampling out and testing colonies grown in the presence of progressively increasing amounts of

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[5] Schmidt, W. H., and Moyer, A. J., *J. Bact.*, 1944, **47**, 199.

[6] Harper, G. J., *Lancet*, 1943, **245**, 569.

TABLE III.

Survival of Penicillinase-producing Staphylococci in Agar Containing Penicillin.

	Growth in penicillin agar		
	Penicillin	Inoculum, per plate	
	Units/ml	5 × 10^7 Colony count*	5 × 10^5 Colony count*
a. *Staphylococcus albus* Shaeffer	0.00	Continuous growth	5 × 10^5
	0.50	,,	10^4
	1.00	,,	2 × 10^3
	2.00	,,	3 × 10^2
	5.00	,,	0
	10.00	10^5	0
b. *Staphylococcus albus* Cleaves	0.00	Continuous growth	5 × 10^5
	0.05	,,	10^4
	0.20	,,	1 × 10^1
	0.50	10^5	5 × 10^{-1}
	1.00	10^4	0
	2.00	1.9 × 10^1	0

* Continuous growth corresponds to more than 1 × 10^6 colonies per plate. Counts higher than 1 × 10^3 are estimates.

TABLE IV.

Growth of Sensitive Staphylococci in Agar and Broth Containing Penicillin.

	Platings in agar		Inoculations in broth Inoculum, bacteria per tube									
Penicillin, units/ml	Survival by colony counts bacteria/ml	Survival ratio	2.8 × 10^7 (a)	2.8 × 10^7 (b)	2.8 × 10^6 (a)	2.8 × 10^6 (b)	2.8 × 10^5 (a)	2.8 × 10^5 (b)	2.8 × 10^4 (a)	2.8 × 10^4 (b)	2.8 × 10^3 (a)	2.8 × 10^3 (b)
0.00	2.8 × 10^8	1	+	+	+	+	+	+	+	+	+	+
0.01	1.0 × 10^6	3.5 × 10^{-2}	+	+	+	+	+	+	+	+	+	+
0.02	5.0 × 10^4	1.8 × 10^{-4}	+	+	+	+	+	+	+	—	—	—
0.04	2.0 × 10^2	7.1 × 10^{-7}	+	+	+	+	—	—	—	—	—	—
0.06	1.3 × 10^2	4.6 × 10^{-7}	+	+	±	+	—	—	—	—	—	—
0.08	3.0 × 10^1	1.1 × 10^{-7}	+	+	—	—	—	—	—	—	—	—
0.10	5 × 10^0	2 × 10^{-8}	—	—	—	—	—	—	—	—	—	—

(a) = predicted; (b) = experimental; + = visible growth; — = no visible growth.

penicillin, results are finally obtained like those shown in Table II, b and c. The strain of Table II, c, appears to be already quite resistant. These results fully confirm those of Demerec.[2]

The data in Tables I and II were obtained by plating suitable numbers of bacteria in agar containing penicillin; in these cases, plates with the same concentration of penicillin and with different inocula give counts proportional to the size of the inoculum. When some of the "resistant" strains isolated from patients are tested, however, the results are often of the type illustrated in Table III. Here the survival, given by colony counts for each concentration of penicillin, is not proportional to the size of the inoculum, but is much higher for heavy inocula.

Each one of the strains giving results of this latter type was found by proper tests to produce penicillinase. Penicillin inactivation was effected both by the whole bacterial cultures and by acetone-ether dried preparations according to Harper.

Results like those recorded in Table III show that the protection afforded by penicillinase is a mass phenomenon, occurring only when bacteria are inoculated in large number. The initial presence of many individuals is probably needed to yield protective amounts of penicillinase. The amounts of extracellular penicillinase carried over with a heavy inoculum are not responsible for the protection, as proved by experiments in which the inoculum consisted of thrice washed cells: the results were similar to those with the whole culture. Moreover, if one uses a small inoculum suspended in the clear centrifugate of a fully grown culture, the amount of survival is not appreciably higher.

It is worth mentioning, incidentally, that in our work we found very little penicillinase activity in the supernatant of penicillinase-producing cultures, and no activity was recovered by filtration through sintered glass, Seitz, or Mandler filters, not even after eluting the filters with phosphate buffer.

An important conclusion from experiments like those represented in Table III is that, when penicillin resistance is of the "penicillinase" type, the individual cells may be much more sensitive than the strain appears to be when tested as a whole. In cases like that illustrated by Table III, b, the individual organisms are almost as sensitive as the cells of a sensitive strain.

Platings in penicillin agar, like those described in Tables I-III, represent a good test for penicillin resistance. In practice, however, the method of quantitative platings is not convenient because of its technical complexity. It was therefore desirable to find out if corresponding results could be obtained in liquid medium.

For bacterial strains that do not produce penicillinase, we may expect that, if the bacteria are inoculated into liquid medium containing various concentrations of penicillin, visible growth will appear whenever the inoculum contains at least one individual resistant to the concentration of penicillin present. By using different inoculum-sizes, it should be possible to estimate the proportion of resistant individuals.

For bacterial strains that produce penicillinase, we may expect visible growth whenever enough penicillinase is produced to destroy the amount of penicillin present. If most cells of the strain are sensitive there should be a large discrepancy between tubes with large and with small inocula. In all cases, therefore, *important inoculum-size effects are to be expected.*

These expectations were fully confirmed by experiments. Table IV shows the results of a typical experiment with a sensitive strain, in which different amounts of bacteria from the same culture were introduced into penicillin broth and into penicillin agar. From the colony counts in agar the expectation of growth in each tube was predicted (columns a) and compared with experimental results (columns b). Predicted and actual results coincide satisfactorily.

Similar results were obtained with all strains not producing penicillinase, whatever their degree of resistance. Visible growth in broth appears whenever the inoculum contains at least one individual resistant to the concentration of penicillin employed.

In similar tests for the strains producing penicillinase, the tubes with heavy inoculum showed growth even with very high concentrations of penicillin; the tubes with small inoculum did or did not show growth depending on the sensitivity of the individual cells, as indicated by independent measurements of survival in agar (Table III).

For the purpose of practical measurement of penicillin resistance, we recommend the following test, which can easily be performed in hospital laboratories and whose results can be read within 24 hours.

A good liquid medium (brain-heart infusion, tryptose broth) is tubed in 5 ml amounts, autoclaved, and stored in the cold. For each test, the tubes are numbered as indicated in Table V. Penicillin is diluted in phosphate buffer before the test to concentration of 5 units per ml (Solution I) and the required amounts are added to the tubes with a 1 ml pipette.

From the culture to be tested, well-grown but not older than 48 hours, 2 successive dilutions (1:100 and 1:10,000) are prepared in 2 tubes of the same medium used for the test (0.05 ml into 5 ml). 0.1 ml of the original culture, or of one of the dilutions, is then introduced into each tube as indicated in the table. For well-grown cultures, the inocula from the undiluted culture and from the two dilutions correspond respectively to 5×10^7, 5×10^5, and 5×10^3 organisms. The tubes are incubated and the results read

TABLE V.
Test for Penicillin Resistance.

Penicillin			a. *Staphylococcus aureus* NRRL Inoculum						b. *Staphylococcus aureus* 26 Inoculum					
Units/ml	Units/tube	Amts of Solution I ml/tube	0.1 ml of culture Tube No.		0.1 ml of dilution 1:100 Tube No.		0.1 ml of dilution 1:10,000 Tube No.		0.1 ml of culture Tube No.		0.1 ml of dilution 1:100 Tube No.		0.1 ml of dilution 1:10,000 Tube No.	
0.00	0.00	0	1	+	2	+	3	+	1	+	2	+	3	+
0.05	0.25	0.05*	4	+	5	—	6	—	4	+	5	+	6	+
0.20	1.00	0.20	7	—	8	—	9	—	7	+	8	+	9	—
0.50	2.50	0.50	10	—	11	—	12	—	10	+	11	+	12	—

* = 1 drop from a 1 ml pipette.

TABLE VI.
Test for Penicillin Resistance.

	Penicillin units/ml	Inoculum 0.1 ml of culture Tube No.		0.1 ml of dilution 1:100 Tube No.		0.1 ml of dilution 1:10,000 Tube No.	
a. *Staphylococcus albus* Shaeffer	0.00	1	+	2	+	3	+
	0.05	4	+	5	+	6	+
	0.20	7	+	8	+	9	+
	0.50	10	+	11	+	12	+
	2.00	13	+	14	±	15	±
	5.00	16	+	17	—	18	—
b. *Staphylococcus albus* Cleaves	0.00	1	+	2	+	3	+
	0.05	4	+	5	+	6	±
	0.20	7	+	8	—	9	—
	0.50	10	+	11	—	12	—
	2.00	13	+	14	—	15	—
	5.00	16	+	17	—	18	—

after 24 hours; a further reading after 48 hours may sometimes show some more positive results.

Table V, a, shows the results for a typical sensitive strain; Table V, b, for a strain of medium resistance; Table VI for two penicillinase-producing strains. For resistant strains we generally repeat the test using amounts of penicillin ten times larger, as shown in Table VI. The amounts of resistant and sensitive cells in each strain are immediately evident by an inspection of the tables.

Discussion. The results described in this paper indicate a definite influence of the size of the inoculum in penicillin sensitivity tests for staphylococcus. The importance of inoculum-size effects for penicillin activity has generally been belittled in the literature on the basis of some early observations, mainly concerned with gross differences in sensitivity.[7]

The inoculum-size effects depends, first, on the presence in sensitive strains of a few mutant individuals of higher resistance; second, on the occurrence of strains producing penicillinase but consisting of cells individually sensitive.

The origin of the penicillinase-producing strains still awaits clarification. Since strains producing large amounts of penicillinase may consist of cells individually sensitive to penicillin, it seems unlikely that penicillinase production be secondarily acquired by resistant strains when grown in presence of penicillin.

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The current tests for penicillin sensitivity[8] do not take into account the inoculum size effects. The use of paper strip tests on heavily inoculated plates is likely to be misleading in cases of relatively sensitive strains producing penicillinase.

A simple test for penicillin resistance is proposed, which indicates the sensitivity of a staphylococcus culture, the presence and proportion of resistant individuals in it, and the degree of their resistance. The test also distinguishes resistant strains from relatively sensitive strains that produce penicillinase. This test may appear to be too delicate because of the fine differences in penicillin sensitivity which it reveals. It must be kept in mind, however, that concentrations of the order of 0.5 units per ml are almost the highest that can be obtained in the blood of patients. A reduction in blood level from 0.5 to 0.2 units per ml in the course of treatment may give a chance for growth to resistant bacterial cells present in a relatively sensitive strain. Moreover, the stepwise increase in bacterial resistance should lead us to consider any strain that is not very sensitive as a potential source of highly resistant variants. The therapeutic use of insufficient concentrations of penicillin may allow these variants to grow and spread, thus nullifying the usefulness of penicillin as a therapeutic agent.

Infections caused by penicillinase-producing strains consisting of individually sensitive cells may be susceptible to penicillin therapy if the number of bacteria present is small enough so that little penicillinase production occurs.

The use of similar tests for bacteria other than staphylococci should await more evidence regarding penicillin-fastness in those organisms.

Summary. The results of penicillin sensitivity tests for staphylococci are influenced by the size of the inoculum on two accounts: (a) the presence in sensitive strains of a minority of resistant individuals originating by mutation; and (b) the occurrence of penicillinase-producing strains whose cells are individually sensitive to penicillin. A simple laboratory test is proposed for the detection and quantitative measurement of penicillin resistance in staphylococcus.

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15223

Effect of Anesthetics and Convulsants on Brain Acetylcholine Content.

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Whether acetylcholine plays an important role in central nervous system synaptic transmission has long been a matter of interest. It is known that central neural tissue contains the ester, can respond to its administration and can synthesize it. Though such facts, taken together with information on peripheral conduction, may suggest a transmitter role for acetylcholine in the central nervous system they do not prove it. Therefore, additional evidence has been sought, and one approach has been to attempt a correlation of acetylcholine content with activity. Results have been varied. Thus, stimulation of afferent nerves has been found to increase production or liberation of acetylcholine by brain,[1,2] but negative results have also been reported.[3,4] Direct faradization

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of the isolated spinal cord of the rabbit[5] and toad,[6] and injection of KCl into the perfused cat brain[7] have been shown to liberate the ester. It has been reported that prolonged strychnine convulsions elevate brain acetylcholine in the frog,[1,8] but that neither strychnine, metrazol nor insulin convulsions raise it in the rabbit.[9] In the rat, on the other hand, there are conflicting reports on the effect of hypoglycaemic convulsions on brain acetylcholine content.[1,10] The effect of light on retinal acetylcholine in the frog has also been studied, and again divergent results have been obtained.[11,12,13]

In a further attempt to correlate brain acetylcholine with activity, we have measured, under constant conditions, the effect of two anesthetics (diffuse diminution of activity) and two convulsants (diffuse increase of activity) on brain acetylcholine content in the rat and frog.

Method. Whole rat brain, including medulla, was placed in a weighed homogenizer tube (Potter-Elvehjem) containing 2 cc of ice cold, eserinized frog Ringer (NaCl 0.67 g, KCl 0.015 g, $CaCl_2$ 0.012 g, $NaHCO_3$ 0.015 g, eserine salicylate 10 mg, and water to 100 cc). The tube was reweighed and homogenization carried out in the original 2 cc from the fourth through the sixth minute after decapitation. After adding eserinized Ringer up to a total volume of 9 cc per gram of tissue, the whole was mixed for one minute. The brei was then divided into two parts. An aliquot of one was assayed for free acetylcholine after centrifugation from the ninth to the twelfth minute at 3000 r.p.m. The remaining brei, acidified to about pH 4 with 0.5 N HCl, was placed in a boiling water bath for 2 minutes. When cool it was neutralized to phenol red, centrifuged, and assayed for total acetylcholine. Frog brain was similarly treated in a smaller homogenizer and made up to a final dilution of 10 cc per gram of tissue. It was assayed without centrifugation for total acetylcholine only.

The assay object was the eserinized, frog, rectus abdominis muscle mounted in a 4 cc muscle chamber. Emphasis has been laid on timing, since we have found that large discrepancies can result from irregular timing.

As we were interested primarily in any measurable relationship between acetylcholine content and activity, animals given convulsants were decapitated as soon as convulsions began. Thus, artefacts due to the anoxia of tonic seizures were avoided.

Results. The data are shown in Tables I and II. On the average, free acetylcholine in rat brain was 67 and 35% higher after chloroform and nembutal anesthesia respectively than in the unanesthetized animal. Total acetylcholine was 35 and 30% higher than without anesthesia. In the frog, total brain acetylcholine was 20% elevated after nembutal. Neither free nor total acetylcholine was elevated in rats or frogs showing convulsive activity after picrotoxin or strychnine.

Our failure to find any effect of strychnine on frog brain acetylcholine conflicts with earlier findings of others[1,8] who have reported an increase. On the other hand, strychnine has been claimed both to have no effect on rabbit brain acetylcholine[1] and to lower it.[14] These conflicting data on the effects of strychnine may in part be the result of different degrees and duration of anoxia developed.[10]

The only data we have seen on the effect of anesthetics were incidentally reported as

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TABLE I.
Effect of Anesthetics and Convulsants on Brain Acetylcholine Content.*

Acetylcholine in whole brain brei, μg per gram.												
Rat										Frog		
Normal†		Chloroform		Nembutal		Picrotoxin		Strychnine		Normal	Nembutal	Strychnine
free	total	free	total	free	total	free	total	free	total	total	total	total
0.7	3.3	1.4	4.4							4.4	5.7	4.8
0.6	3.0	0.9	3.9							5.4	6.3	6.6
0.6	3.5	1.2	4.5							4.5	5.3	4.9
0.6	2.1	0.8	2.7			0.6	2.5			5.4	6.2	4.8
0.6		0.9								5.0	5.8	4.6
0.6		0.8										
0.7		0.9										
0.5		0.9										
0.5		0.9										
0.6	3.7			0.9	4.9							
0.7	3.7			0.9	5.2							
0.7	3.3			1.0	3.9							
1.0	2.9			1.3	3.6	0.9	2.2	1.0	2.5			
1.0	3.1			1.5	3.3	1.0	2.0	1.1	2.6			
1.0	2.4					1.0	2.2					
0.7	2.3					0.4	2.2					
0.6	2.4							0.5	2.4			
0.5	2.5							0.9	2.4			
0.6	2.8							0.5	2.6			
—	—							0.6	2.8			

* Data on experimental animals are tabulated opposite controls run simultaneously.
† Average normal values, rat: free 0.7 (S.E. 0.04); total 2.9 (S.E. 0.14).

TABLE II.
Effect of Anesthetics and Convulsants on Brain Acetylcholine Content.
Average Data and Statistical Significance.*

Drug		Animals	Avg acetylcholine content, γ/g			
			Free		Total	
			content	difference	content	difference
Chloroform	10-60 min. anesthesia	normal rats	0.6 ± 0.02		2.9 ± 0.31	
		anesthetized rats	1.0 ± 0.06	0.4 ± 0.07	3.9 ± 0.41	1.0 ± 0.51
Nembutal	30 mg/kg. 10-60 min. anesthesia	normal rats	0.8 ± 0.08		3.3 ± 0.15	
		anesthetized rats	1.1 ± 0.12	0.3 ± 0.15	4.2 ± 0.37	0.9 ± 0.40
Picrotoxin	20 mg/kg	normal rats	0.9 ± 0.09		2.6 ± 0.19	
		convulsant rats	0.8 ±0.12	0.1 ± 0.07	2.3 ± 0.09	0.3 ± 0.21
Strychnine	3 mg/kg	normal rats	0.7 ± 0.11		2.7 ± 0.13	
		convulsant rats	0.8 ± 0.11	0.1 ± 0.11	2.6 ± 0.07	0.1 ± 0.15
Nembutal	30 mg/kg	normal frogs	—	—	4.9 ± 0.21	
		anesthetized frogs	—	—	5.9 ± 0.18	1.0 ± 0.28
Strychnine	3 mg/kg	normal frogs	—	—	4.9 ± 0.21	
		convulsant frogs	—	—	5.1 ± 0.11	0.3 ± 0.27

* Statistical significance is indicated as ±S.E., not as ±P.E.

part of a different type of investigation.[10] Three nembutalized rats yielded an average value of 0.35 or 0.43 (depending upon method) micrograms of acetylcholine per gram of brain, whereas the figure for 7 normals was 0.25 or 0.35 μg per gram. Here the direction and magnitude of difference agree with our findings.

In general, our values for free acetylcholine in the rat brain are somewhat higher than those reported elsewhere.[10] Preliminary experiments lead us to believe that the difference is probably a result of more efficient extraction because of finer homogenization obtained with the Potter-Elvehjem homogenizer than with the sand-in-mortar type used by others.

It is not yet possible to define rigorously the physiological meaning of these results. One might assume that chloroform and nembutal raised brain acetylcholine above a "normal" level, but it is equally likely that the values obtained during anesthesia more nearly approximate the "true" resting levels. Thus greater activity, whether that of the usual waking state or that of the more active, convulsive state, is paralleled by a lower brain acetylcholine content than is rest. Whether this reflects an increased rate of acetylcholine breakdown during activity cannot be said. The change observed might have been much greater if brain regions rather than whole brain had been assayed. As the experiments were done, a large change in a small region may have been masked by the diluent effect of a large volume of tissue in which acetylcholine content did not change.

It will be recalled that chloroform liberates free acetylcholine from bound precursor *in vitro*.[15] For this reason we also used the quite different type of anesthetic, nembutal. In two experiments *in vitro*, nembutal did not liberate free from bound acetylcholine in homogenized rat brain. For this reason, and because bound acetylcholine was actually increased more than the free after choloroform or nembutal, our *in vivo* results probably do not reflect simple liberation of free ester from bound precursor. It is always possible that acetylcholine may accumulate as a result of esterase inhibition. Certain depressants have been shown to have anticholinesterase activity,[2,16,17] but this is apparently not the case with chloroform or single large doses of barbiturate.[18]

Conclusions. 1. Both free and total acetylcholine content of the whole rat brain are higher after nembutal or chloroform anesthesia (diffuse diminution of activity) than in the unanesthetized animal. The free acetylcholine change is more convincing after chloroform, whereas the total acetylcholine change is more convincing after nembutal. The total acetylcholine content of the frog brain is similarly elevated after nembutal.

2. Neither free nor total acetylcholine content of rat brain differed significantly after the onset of strychnine or picrotoxin convulsions (diffuse increase of activity) from that found in quiet animals awake. Nor did strychnine alter total acetylcholine content of the frog brain.

3. With the methods used, normal whole rat brain has been found to contain about 0.7 μg of free and 2.9 μg of total acetylcholine per gram wet weight; frog brain about 4.9 μg of total acetylcholine per gram.

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15224

Effect of Nutrition on Susceptibility of Mice to Pneumococcal Infection.

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Several workers have studied the effects of various diets on the susceptibility of mice and rats to induced pneumococcal infections.[1,2,3,4] For the most part, these studies have dealt with the effects of B vitamin deficiencies. The observed differences between control and experimental animals have been small, and not altogether consistent from laboratory to laboratory.

It has been found in these laboratories

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TABLE I.
Effect of Diet and Concentration of Inoculum on the Resistance of Mice to Pneumococcal Infection.

Diet	Culture conc.	No. of mice	No. surviving at day indicated 1	2	3	4	6	Avg survival days	% survival
A*	10^{-8}	24	24	20	16	15	13	4.2	54
	10^{-7}	10	10	3	3	2	1	2.0	10
	10^{-6}	56	56	18	11	10	6	1.91	11
	10^{-5}	10	10	1	0	—	—	1.1	0
B†	10^{-6}	10	10	6	4	4	4	3.2	40
C‡	10^{-6}	10	8	8	6	6	3	3.4	30
Basal	10^{-6}	52	52	50	48	46	45	5.50	86
	10^{-5}	9	9	9	8	7	7	5.2	78
	10^{-4}	14	14	13	13	13	13	5.6	93
	10^{-3}	9	8	7	5	5	5	3.8	56
	10^{-2}	10	7	3	3	3	3	2.2	30

* Purina Fox Chow.
† Rockland Mouse Diet.
‡ Stock diet of Robinson and Siegel.[2]

TABLE II.
Duration of Dietary Regimen and Change in Susceptibility of Mice to Pneumococcal Infection.

Days on basal diet*	No. of mice	No. surviving 6 days	Avg survival time, days
0	10	4	3.0
1	10	6	4.5
4	10	8	5.2
5-16	52	45	5.5

* Previous to injection. After injection all animals were maintained on the basal diet.
Dose = 10^{-6} ml culture per mouse.

that a tremendous difference in susceptibility to pneumococcal infection exists between mice which have been fed a purified diet and mice maintained on a commercial small-animal diet.

Albino mice (18 to 24 g) were injected intraperitoneally with 0.5 ml of a dilution of a 17 hour culture representing 1.0 ml of a 10^{-6} concentration. The actual number of organisms injected varied from 235 to 700 as determined by plate counts. The pneumococcus, Type I, SV-1 strain, was grown on proteose-peptone-blood broth, and was passed through mice at weekly or biweekly intervals.

The basal diet consisted of casein 180, salts 20, cotton-seed oil 30, cod liver oil 20 and sucrose 750 g, supplemented by thiamin chloride hydrochloride 5, riboflavin 5, pyridoxine chloride 2, calcium pantothenate 20, nicotinamide 40, p-aminobenzoic acid 20, 2,3-dimethyl-1,4-naphthoquinone 0.2 and α-tocopherol 1 mg and choline chloride 1 g per kilo of diet respectively.

Mice not on experiment were maintained on commercial diet A (Table I). Experimental mice usually were fed the diet to be tested for 5 days before the injection of the pneumococci. Experimental animals were observed over a 6 day period, and the results were recorded in terms of average survival time (maximum 6.0).

Results. Table I shows the effect of various dosages of pneumococcus culture on the production of infection in mice which had been maintained on different diets. It is seen that on diet A the mice are quite susceptible to infection, and that it is only when the culture is very dilute (10^{-8}) with an average dose of 3.5 organisms, that any considerable proportion survives the injection. A control group, maintained on this diet, was injected with the culture used for each experiment. The data of Table I (dose 10^{-6} ml) represent the summation of 7 experiments with groups of 5 to 10 mice which gave average survival times ranging from 1.4 to 3.0 days and from 0 to 20% survival at the termination of the experiment. On diet B the mice appear to be somewhat less sus-

TABLE III.
Protocol of Exp. 5, May 29, 1945.

Diet	No. of mice	No. surviving at day indicated 1	2	3	4	6	Avg survival, days
A*	10	10	1	1	1	0	1.4
Basal	10	10	10	8	8	7	5.0
Biotin, inositol, folic acid	10	10	10	9	9	9	5.6
10% brewer's yeast	10	10	9	9	8	5	4.6
5% 70 A.I. liver	10	10	9	7	7	6	4.5
Alcoholic extract of liver	10	10	10	9	9	9	5.6

* Purina Fox Chow.

ceptible; the average survival time, 3.2 days, is greater than any found with the same dosage for a group of 10 mice on diet A. Mice on the stock diet of Robinson and Siegel[2] are less susceptible but significantly more susceptible than those on the purified diet.

Mice maintained on the basal diet are very resistant to pneumococcal infection. Thus it was only when the concentration of pneumococcus culture reached 10^{-2}, the equivalent of about one million lethal doses for mice on diet A, that any considerable proportion succumbed to the infection. A control group of 5 to 10 mice, maintained on basal diet, was injected with the culture used for each experiment. The data of Table I (dose 10^{-6} ml) represent the summation of 8 such experiments which gave average survival times ranging from 4.4 to 6.0 days and from 60 to 100% survival.

The protocol of a representative experiment is given on Table III. All animals were injected with 0.5 ml aliquots of the same culture dilution representing 10^{-6} ml of a 17 hour blood broth culture. Animals were caged in groups of 5, with 2 groups on each diet. Injections were completed within 45 minutes. However, to distribute evenly the effect of any possible change in the culture during this period, one group of five on each of the diets was inoculated before the second group on any diet was injected. The composition of the diets is given in Table IV.

The time of pretreatment on the basal diet to effect a change in susceptibility was determined in the experiments reported in Table II. It appears that if the mice are changed from diet A to the basal diet at the time of injection some increased resistance to the infection may result, while even a single day on the basal diet definitely increases the resistance.

In Table III are shown the results of a number of experiments designed to test the effect of various dietary supplements to the basal diet. In every instance the average survival time of the group of mice fell within the range of survival times found for groups of 5 to 10 mice on the basal diet. In only one instance, that of the diet in which 5% of the 70% alcoholic insoluble fraction of liver was incorporated, was the average survival time low enough to indicate that further investigation would be desirable. It is apparent that the difference between the basal and the commercial diet cannot be attributed to any known vitamin or, in fact, to any known dietary essential.

Discussion. A number of possible explanations exist for the difference in susceptibility to pneumococcal infection of mice on the two types of diet. These theories fall into two main groups. Either a protective factor in the purified diet or a factor in the commercial diet which increases the susceptibility might be postulated. The first appears unlikely. The latter explanation would be best fitted to explain the results to date. On this hypothesis some factor essential to the rapid multiplication of the pneumococcus, but not necessarily an absolute requirement for growth, would be absent from, or in low concentration in, the synthetic diet. This hypothetical substance (or substances), would not be essential for the mouse. It would, in fact, be present in minimal concentration in the blood and body fluids of

TABLE IV.
Effect of Various Supplements to the Purified Diet on Susceptibility of Mice to Pneumococcal Infection.

Supplement to basal diet	No. of mice	No. surviving 6-days	Avg survival time, days	% survival
Biotin, inositol[*]	5	5	6.0	100
Biotin, inositol, folic acid[†]	20	18	5.7	90
Alcoholic extr. of liver[‡]	10	9	5.6	90
75% corn starch	11	8	5.2	73
3% salts	6	6	6.0	100
12% casein	10	8	5.3	80
12% proteose-peptone	10	9	5.7	90
2% 70 A.I. liver[§]	10	10	6.0	100
5% 70 A.I. liver	10	6	4.5	60
10% brewer's yeast	20	15	5.3	75
5% asparagine	10	10	6.0	100

Each supplement was incorporated into the basal diet by replacement of an equal weight of sucrose.

[*] 40 mg inositol, 400 μg biotin per kg diet.

[†] 2 mg biotin, 80 mg inositol, and 15 g folic acid concentrate (equivalent to 6 mg of folic acid $\pi = 40,000$) per kg diet.

[‡] Fraction soluble in 70% alcohol equivalent to 0.8 g liver per g diet.

[§] Fraction insoluble in 70% alcohol.

Dose = 10^{-6} ml culture per mouse.

the mouse, except insofar as higher levels were maintained by a more or less continuous supply from exogenous sources. Such an hypothesis would explain the rapid change in susceptibility of the mouse which occurs when the dietary regimen is changed.

The conclusions to be drawn from the present experiments may or may not be applicable to other types and strains of pneumococci. However, Robinson and Siegel[2] found mice on all purified diets to be less susceptible to pneumococcal infection than those on their stock diet. The data reported here show similar comparative effects from their stock diet and the purified diets. It seems likely, therefore, that the same factors influencing susceptibility may be operative in both laboratories.

Summary. The resistance of mice to the intraperitoneal injection of Type I pneumococcus was found to be markedly dependent on the type of diet on which they were maintained. Those on a purified diet survived the equivalent of 100,000 lethal doses for those on a commercial laboratory diet. This difference could not be ascribed to any known dietary essential.

We are indebted to Armour Laboratories for generous supplies of alcoholic liver extract and folic acid concentrate, and to Dr. Marion B. Sherwood for plate counts, dilutions, and the care of cultures.

15225

Further Purification of the Follicle-Stimulating Hormone and Its Effect in Normal and Hypophysectomized Rats.[*]

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Follicle-stimulating hormone preparations have been obtained previously by the digestion of an aqueous extract of sheep pituitary powder with trypsin.[1,2] The extracts obtained by this method were biologically tested mainly by use of normal immature female rats, although the effect of a few of these preparations in hypophysectomized rats was reported.[1] The preparations obtained by the method reported in 1940[2] when used in the human being caused local reactions at the site of injection, which made it necessary to do further work in order to remove this local reacting factor. This report is concerned with the method of removal of this factor, and with the assay of several preparations in normal and hypophysectomized rats.

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[*] Supported in part by a grant from the Wisconsin Alumni Research Foundation, and in part by grants from Eli Lilly and Company and The Abbott Laboratories.

[1] McShan, W. H., and Meyer, Roland K., *J. Biol. Chem.*, 1938, **126**, 361.

[2] McShan, W. H., and Meyer, Roland K., *J. Biol. Chem.*, 1940, **135**, 473.

Experimental. Removal of local reacting factor. The procedure followed is the same as that reported previously[2] until the fraction of the digest which is soluble at pH 4 is obtained. Instead of precipitating the active material at this stage with ethyl alcohol, it was dialyzed in the cold against distilled water which was changed 5 times. This caused the formation of a light inactive precipitate which was separated by centrifuging. The hormone was precipitated from the solution by addition of 4 volumes of 95% alcohol. Precipitation was aided by addition of 4 drops of 2N HCl while the mixture was being stirred.

The precipitate was recovered by centrifuging and the active material was recovered by extracting the precipitate with cold distilled water. It was necessary to make at least 5 extractions using 75 ml of water each time for each 200 g of pituitary powder. Thorough extraction was accomplished by shaking the mixture with glass beads. During the third extraction the insoluble residue became gummy so that it was necessary to break the insoluble particles with a glass rod. Each extraction was continued for 2 to 4 hours followed by centrifuging and decantation of the extract into 1.5 liters of cold acetone. After each extraction the extract was added to the acetone to which the others had been mixed. A few drops of 2N HCl were added followed by stirring to cause precipitation after which the mixture was allowed to stand overnight in the cold.

The precipitate was collected by centrifuging. It was dried either by washing with acetone, or by lyophilizing after solution in 0.02 M phosphate buffer of pH 7.4. The product obtained by either of these methods of drying is soluble in water, or in 0.9% saline and can be sterilized by Seitz filtration.

Assay—Normal rats. The total amount of the hormone preparation to be given to a single rat was dissolved in 4.5 ml of 0.9% sodium chloride. Solutions of this kind were injected subcutaneously into 21 day old female rats in nine injections of 0.5 ml each beginning on the afternoon of the first day and followed by morning and afternoon injections for the next 4 days. Autopsy was done during the morning of the sixth day. The ovaries were removed, examined grossly with the aid of transmitted light for the presence of follicles and corpora lutea, and weighed. These ovaries were prepared for microscopic study and later they were examined for the presence of lutein tissue.

Hypophysectomized rats. Immature female rats 28 days old were obtained from Sprague-Dawley, Inc. These animals were hypophysectomized on the 29th day, and the injections were started the following day when they were 30 days old. The total amount of hormone to be given each rat, which was usually 2.5 g,[†] was dissolved in 10 ml of 0.9% sodium chloride. They were injected subcutaneously with 0.5 ml on the morning and afternoon of each day for 10 days, and were killed on the morning of the eleventh day at which time the ovaries were removed, examined, weighed and prepared for histological study. Completeness of hypophysectomy was determined at the time of autopsy by use of a binocular microscope.

Results and Discussion. Assay of preparations that contained the local reacting factor in normal and hypophysectomized rats. The 10 preparations listed in Table I were prepared according to the tryptic digestion method reported in 1940,[2] and thus contained the local reacting factor. Five hun-

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[†] Indicates in all cases grams equivalent of original pituitary powder used in making the preparation.

TABLE I.
Assay of FSH Preparations Which Caused Local Reactions in the Human Female.*

Prep. No.	Mg per g. eq.	Total‡ dose, g	No. of rats	Wt of ovaries, avg mg	Qualitative response: Ovaries	Qualitative response: Vagina
		Normal rats injected 4.5 days.				
5000†	11.57	0.5	6	85	follicles only	O§
508	15.10	0.5	6	56	" "	No
514	—	0.5	3	27	" "	No
		1.0	3	99	" "	O
515	13.50	0.5	3	32	" "	No
516	12.40	0.5	6	50	" "	O
517	8.33	0.5	6	62	" "	O
519	—	0.5	6	105	" "	O
520	—	0.5	3	38	" "	No
		1.0	3	100	" "	O
522	11 80	0.5	7	41	" "	No
523	13.24	0.5	3	110	corpora	O
Avg	12.13					
		Hypophysectomized rats injected 10 days				
5000†	11.57	2.5	4	183	follicles only	O
508	15.10	2.5	5	232	" and few bl. pts.	O
514	—	2.5	5	65	" only	O
516	12.40	2.5	5	263	" "	O

* Made by method reported in 1940.[2]

† FSH5000 was made by combining parts of preparations 508, 510, 513, 515, 516, and 517.

‡ Indicates g equivalent of pituitary powder.

§ O indicates that vagina was open; No that vagina was not open.

dred grams of pituitary powder was used in making each of these preparations. The total dose used for assay in normal rats in most cases was 0.5 g. The ovarian weights obtained by injecting this amount of hormone weighed from 27 to 110 mg and the average was 61 mg. These ovaries, with the exception of those produced by preparation 523, contained follicles only as indicated by microscopic examination at the time of autopsy. The uteri were highly distended in all cases, and certain of the preparations caused the vaginas to open. The fact that preparation 523 produced ovaries that contained corpora lutea suggests that the extract was not properly digested. The failure of the extract to clear during digestion as it usually does, further supports this idea, and emphasizes the need for making a systematic study to determine the optimum conditions for digestion.

Preparations 5000, 508, 514 and 516 were tested in hypophysectomized rats by administering a total dose of 2.5 g over a period of 10 days. The ovaries obtained ranged in weight from 65 to 263 mg; the average weight was 186 mg. The uteri were distended with fluid and the vaginas were open in all cases. Macroscopic examination of these ovaries showed that they contained only follicles with the exception of those from one rat injected with FSH508 which contained a few hemorrhagic follicles.

It was found on microscopic examination that certain of these ovaries contained several patches of hypertrophied theca cells. One ovary contained some lutein cells, and there were a total of 8 small or partly formed corpora in four ovaries. Thus these preparations as indicated by assays in hypophysectomized rats contained very little luteinizing hormone but evidently were not completely devoid of luteinizing activity.

Assay of preparations that were free of the local reacting factor in normal and hypophysectomized rats. The results of the assays in normal rats of 15 different follicle stimulating preparations that were essentially free of the local reacting factor, are given in Table II. These preparations were tested by administration of 0.5 g and 1 g total doses. The ovaries as indicated by macroscopic examination at the time of autopsy contained only follicles with the exception of 3 pairs produced by 3 different preparations each given in a total dose of one gram. The

TABLE II.
Results of the Assay in Normal and Hypophysectomized Rats of Follicle-stimulating Preparations.*

				Qualitative response	
Prep. No.	Total dose, g. eq.	No. of rats	Wt of ovaries	Ovaries	Vagina
230	1.0	3	87	follicles only	O†
234	0.5	6	38	” ”	No
234	1.0	6	90	” ”	O
235	0.5	6	50	” ”	O
235	1.0	3	90	” ”	O
236	0.5	3	68	” ”	O
236	1.0	3	90	” ” (few CL in 1 pair ov.)	O
237	0.5	6	35	follicles only	No
320	0.5	3	42	” ”	No
321	0.5	3	35	” ”	No
321	1.0	3	104	” ”	No
322	0.5	6	46	” ”	No
322	1.0	3	55	” ”	No
323	0.5	3	34	” ”	No
323	1.0	9	60	” ” (1 pair ov. weighed 114 mg, contained few CL)	No
327	0.5	6	61	follicles only	No
327	1.0	3	102	” ” (few CL in 1 pair ov.)	O
328	0.5	9	32	follicles only	O
328	1.0	15	75	” ”	O
330A	0.5	3	41	” ”	
330B	0.25	6	34	” ”	No
330B	0.5	9	68	” ”	O
331	0.5	3	36	” ”	No
331	1.0	3	69	” ”	No
333	0.5	6	25	” ”	No
333	1.0	7	57	” ”	No
Hypophysectomized Rats. (2.5 g given over 10 days.)					
333	2.5	4	75	follicles only	O

* These preparations were made after the method was changed to remove the local reacting factor, and several of them were administered to human clinical cases.[3]

† O indicates that vagina was open; No that vagina was not open.

uteri were usually distended with fluid and in many cases the vaginas were not open. It is of interest to note that 0.25 g of FSH330B produced ovaries that had an average weight of 34 mg which was almost as great as that obtained with 0.5 g of the other preparations. This difference is believed to be due to the fact that the aqueous extract used in making FSH330B was not supercentrifuged before it was digested with trypsin. This suggests that more active preparations may be obtained without supercentrifuging but further work is necessary to test this possibility. Several of these preparations were effective in stimulating the ovaries of the human female, and in addition they caused little trouble from the standpoint of producing local reactions at the injection sites.[3]

FSH333 was assayed by use of hypophysectomized rats. The average weight of the ovaries was 75 mg and they consisted of follicles only as indicated by observation at the time of autopsy (Table II). These ovaries were sectioned and examined microscopically. One pair of ovaries contained no lutein cells; another pair showed one small group of hypertrophied theca interna or granulosa cells; the third pair contained 3 small groups of lutein cells; and the fourth pair had 5 very small corpora. Thus it is clear that this preparation contained very little luteinizing activity as indicated by the administration of a total dose of 2.5 g over a period of 10 days. This demonstrates that follicle-stimulating preparations of a high degree of physiological purity can be obtained by the tryptic digestion method as described in this report.

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The criteria used in assaying follicle-stimulating preparations obtained by the tryptic digestion method are relatively large increases in the weights of the ovaries of normal and hypophysectomized rats without the production of any lutein tissue or discrete corpora lutea. If these rigid specifications are to be the basis for determining the absolute purity of follicle-stimulating preparations, then not all preparations obtained by tryptic digestion are physiologically pure. These preparations are suitable for many practical purposes as demonstrated by their use in human beings,[3] and in laboratory and domestic animals.[4,5,6] The large doses and long injection periods used in testing the preparations are believed to be suitable for the maximum expression of any small amount of luteinizer that might be present in the preparations. When intact immature rats were used for assay most of our preparations have produced ovaries that contained only follicles as indicated by macroscopic examination. We believe that results of this kind, when obtained consistently, indicate that this method in which intact rats are used is as sensitive for detecting small amounts of luteinizer in the presence of follicle-stimulating hormone as are hypophysectomized rats. This is because the intact rat has a normal endocrine balance and the secretion of luteinizer by the animal's own pituitary gland would be added to any traces present in the follicle-stimulating preparation injected. If, under these conditions, a preparation produces ovaries that contain only follicles, it is believed the preparation has a high degree of physiological purity.

The product obtained by the tryptic digestion process is undoubtedly different chemically from preparations prepared by other methods such as salt fractionation reported by Greep *et al.*[7,8] It is also probable that the product obtained by the method developed by these workers is different in its physiological action from that obtained by tryptic digestion as little increase in ovarian weight resulted and no secretion of estrogen was obtained, even when large doses were given to hypophysectomized rats.[9] Another difference is that these workers used fresh hog pituitary glands in their work on chemical fractionation while whole sheep pituitary powder was used in the tryptic digestion method. Further study is needed to determine whether these variables are responsible for the different physiological effects produced by the two kinds of preparations.

Summary. A method is described for the preparation of follicle-stimulating preparations by tryptic digestion and the removal of a fraction which caused reactions in the human being at the site of injection.

Preparations made by the tryptic digestion method before and after it was changed to remove the local reacting factor were assayed both in normal and hypophysectomized rats. By macroscopic observation the ovaries from both kinds of rats appeared to contain only follicles but microscopic examination showed that a few contained lutein tissue indicating that not all the preparations were entirely devoid of luteinizing activity.

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15226

Urinary Excretion of Estrogens and 17-Ketosteroids in Young, Adult Males With Infectious Hepatitis.

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Male subjects with chronic diseases of the liver frequently display symptoms which are best explained by assuming an increased concentration of circulating, biologically active estrogenic compounds. Testicular atrophy,[1] gynecomastia, decrease in total body hair, development of spider telangectasia, feminine distribution of pubic hair, and loss of libido,[2,3,4] include some of the phenomena associated with cirrhosis of the liver in the male. In some of these cases a high output of urinary estrogen has been recorded, along with a fall in the total excretion of urinary androgens.

Numerous experiments with animals have shown that the liver inactivates, or otherwise disposes of large quantities of exogenous and endogenous estrogens.[5–11] If estrogenic compounds are injected into the portal circulation, their systemic effects are negligible compared to the effect of a similar quantity of hormone injected into extra-portal areas.[12,13,14] At least 2 major roles have been assigned to the liver in the metabolism of estrone; one, that it is rapidly effective in converting estrone to estradiol, and secondly, that it acts to convert all estrogenic materials to compounds which are no longer phenolic in character.[9] These data provide a theoretical basis for explanation of the marked degree of feminization occasionally seen in males with advanced cirrhosis of the liver[15,16] and suggest that estrogen metabolism may be significantly impaired in chronic hepatic insufficiency.

In the present study an attempt has been made to learn whether defective estrogen metabolism likewise occurs in acute infectious hepatitis. At the same time studies on the urinary excretion of the total 17-ketosteroids have been carried out in order to learn if the metabolism of androgens may also be affected in acute liver insufficiency. The results of serial studies of the urinary excretion of estrogens and 17-ketosteroids in 11 males with infectious hepatitis are recorded in the accompanying graph (Fig. 1). All patients selected for this study were young adult males, previously in good health, and with no demonstrable diseases other than hepatitis. The number of days following the appearance of the first symptoms are recorded on the abscissa. The concentration of free urinary estrogen and total 17-ketosteroids, in micrograms and milligrams per 24 hours, respec-

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* The authors wish to acknowledge the valuable technical assistance of Miss Elizabeth Van Pelt.

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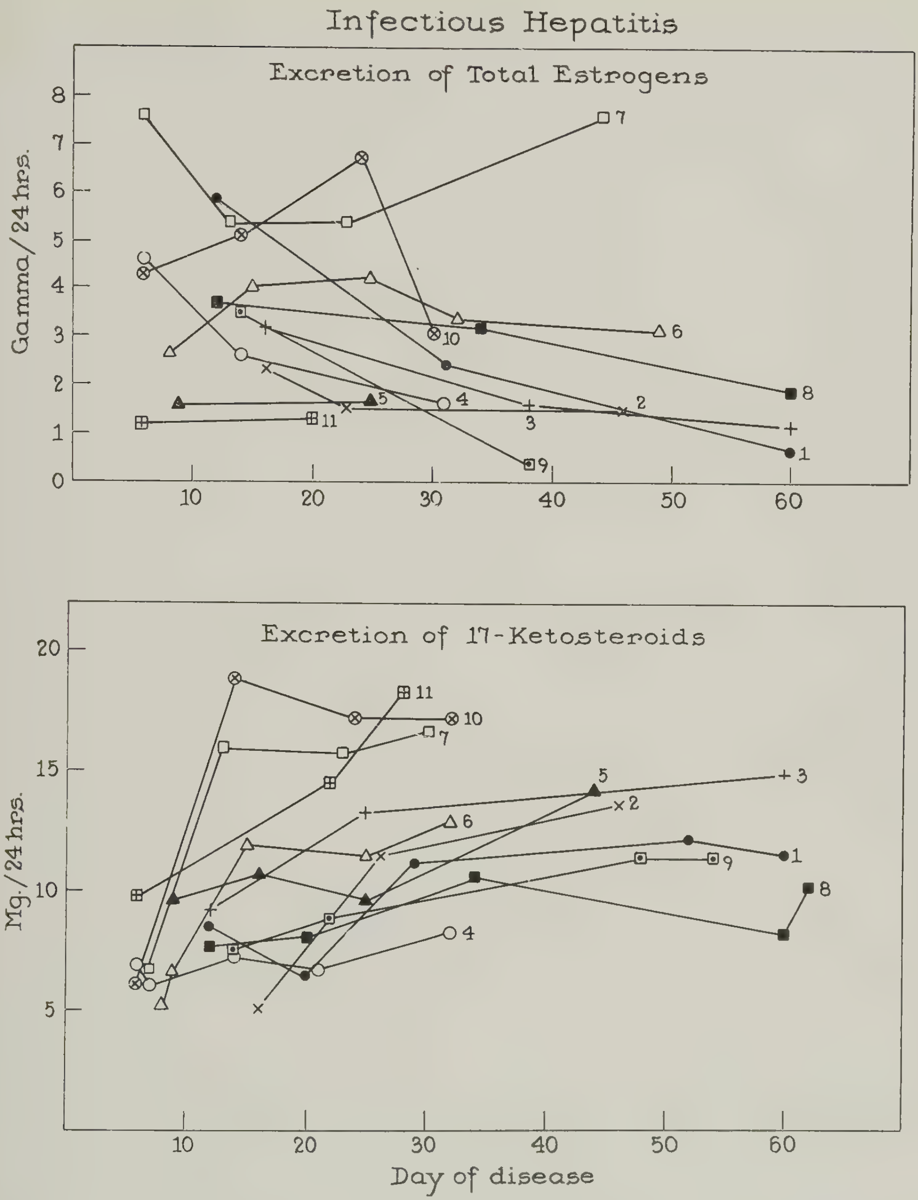

FIG. 1.

Graph showing values for the urinary excretion of estrogens and 17-ketosteroids in young, adult males with infectious hepatitis.

tively, has been plotted on the ordinate. The estrogen assays were carried out by a modification of the Allen-Doisy technic,[17,18] in which 0.1 cc of a urine extract prepared by hydrolysis, extraction with carbon tetrachloride, and concentration of the phenolic compounds in the standard manner, was injected 4 times in 48 hours into spayed mice, and vaginal smears studied on the third day. An average of 15 animals were used for each urine extract. The method for the 17-ketosteroids was essentially

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that of Talbot,[19] with modifications suggested by Dr. Ephraim Shorr.[20] In general the methods proved to be reliable, and the results reproducible.

The range of excretion of estrogens in normal males is low, and quite varied. Moreover, the values depend to a great extent upon the technic employed for the preparation of the urinary extract and upon the method used for assay. It was decided, therefore, to follow the estrogen excretion of each patient at frequent intervals throughout the course of the disease, and to use as a control base line for each case the quantity of estrogenic substances excreted following recovery from hepatitis.

With the convalescent level as a control, it may be seen that in nearly every case there was a significant rise in estrogen excretion during the acute phase of hepatitis, and that a diminishing elevation in estrogen excretion was maintained until relatively late in convalescence. In Subject 7, values for the urinary excretion of estrogens were high within the first 5 days following the onset of the disease process. By the 12th day an abrupt fall had occurred, associated with marked clinical improvement. No further drop in estrogen excretion was observed. However, on the 45th day after onset of the disease, a significant rise in the excretion of urinary estrogens had again occurred, coincident with a return of clinical signs of hepatitis. Following clinical recovery of the patient, normal values were recorded for the urinary excretion of estrogens of 2.9 μg per 24 hours. Delayed clinical recovery in another patient with hepatitis, Case No. 6, was likewise reflected in a persistent moderate elevation of urinary estrogens. Further studies, attempting to relate persistent elevation in urinary estrogen excretion to delay in convalescence and chronicity in infectious hepatitis are under way, and the results will be published subsequently.

In most cases there appeared to be a significant correlation between the severity of the attack of acute hepatic insufficiency, as judged from the results of clinical and laboratory studies, and the estrogenic activity of the urine. For example, Case No. 5 and No. 11, in whom normal values for the urinary excretion of estrogens were recorded during the course of the disease, presented exceptionally mild symptoms of infectious hepatitis, requiring only 20 and 28 days, respectively, for complete clinical recovery.

The normal range of the urinary level of 17-ketosteroids in males, like that for the estrogens, is variable, with most reports placing values at 8-28 mg per 24-hour period of urinary excretion. Again, because of the wide variation in the normal level of androgen excretion, it was considered that the convalescent level of 17-ketosteroids provided the best possible normal base line for each patient, and the most effective standard for a comparison of the degree of aberration in 17-ketosteroid excretion occurring in each case during the acute and convalescent states of infectious hepatitis.

The urinary excretion of 17-ketosteroids in the majority of cases was low in the early stages of hepatitis, and rose to a normal level during the period from the 20th to the 50th day of the disease, where it remained approximately constant. In some cases the level of excretion was more than twice the value obtained during the acute stages of the disease. In 2 cases in whom there occurred recrudescence of hepatitis with a return of clinical symptoms, the 17-ketosteroid levels of the urine fell again to values comparable to those observed during the first acute attack of the disease. In both cases clinical recovery was followed by an elevation of the urinary 17-ketosteroids to normal values.

At first glance there appears to have been a reciprocal relationship between the levels of excretion of estrogens and 17-ketosteroids in the urine of patients with acute hepatic insufficiency. Closer analysis, however, reveals that in all cases, a rise in 17-ketosteroids, often to a normal level, occurred considerably in advance of a significant decrease in the level of urinary estrogens. The excretion of 17-ketosteroids is temporarily depressed in a variety of conditions, including fasting, protein depletion, and general debility. It is quite possible, therefore, that the depression of the excretion of 17-ketosteroids observed in acute hepatic insufficiency may have resulted from the secondary effects of the disease, rather than to a rise in the level of active circulating estrogens. That reciprocal depression of androgen formation may occur eventually, as a result of the failure of the liver to accomplish the inactivation of estrogenic compounds, is a definite possibility, and may conceivably account for the persistent diminution in the excretion of 17-ketosteroids which has been observed in chronic hepatic insufficiency.

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Summary. The levels of excretion of estrogens and 17-ketosteroids in the urine have been determined in 11 young adult males with acute infectious hepatitis, at frequent intervals, from onset of the disease to convalescence. In 9 moderately severe cases there was a significant elevation in the level of excretion of biologically active estrogens in the urine which persisted at a slowly diminishing elevation until convalescence was considerably advanced. There was no significant increase in the concentration of the urinary estrogens in 2 cases of hepatitis which presented only mild clinical symptoms.

Marked to moderate depression occurred in the level of excretion of urinary 17-ketosteroids early in the course of acute hepatitis, with a return to normal values considerably in advance of the period of diminished estrogen excretion and of clinical recovery.

15227

Adequacy of the Known Synthetic Vitamins for Normal Feathering and Pigmentation in Chicks.

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From the Abbott Laboratories, North Chicago, Ill.

The existence of a factor, or factors, in liver or yeast other than pantothenic acid, *p*-aminobenzoic acid, biotin, or inositol necessary for normal pigmentation was indicated from earlier work in this laboratory with rats[1,2] and dogs.[3] McGinnis, Norris, and Heuser[4] likewise indicated the presence of an unidentified factor other than pantothenic acid in brewers' yeast which was necessary for normal feathering and pigmentation in growing Rhode Island Red chicks. Cure or prevention of the achromotrichia developed in rats fed sulfaguanidine or succinylsulfathiazole by administration of folic acid concentrates was reported by Martin[5] and by Wright and Welch.[6] Similar results have been obtained in this laboratory using liver concentrates. It appeared, therefore, that the active factor might be identified with the *L. casei* factor[7] and vitamin B_c.[8]

Attempts have been made to develop techniques capable of assay for the unknown pigmentation factor. Chicks and dogs on highly purified diet and rats on purified diet with added succinylsulfathiazole have been used. Most consistent results have been obtained with growing chicks. In studying the role of the *L. casei* factor in hair and feather pigmentation, concentrates of the vitamin prepared by one of us (F.C.M.) and finally the

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[1] Frost, D. V., Moore, R., and Dann, F. P., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 507.

[2] Dann, F. P., Moore, R., and Frost, D. V., *Fed. Proc. Soc. Exp. Biol. and Med.*, 1942, **1**, 107.

[3] Frost, D. V., and Dann, F. P., *J. Nutrition*, 1944, **27**, 355.

[4] McGinnis, J., Norris, L. C., and Heuser, G. F., *J. Biol. Chem.*, 1942, **145**, 341.

[5] Martin, G., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 353.

[6] Wright, L. D., and Welch, A. D., *J. Nutrition*, 1944, **27**, 55.

[7] Pfiffner, J. J., *et al.*, *Science*, 1943, **97**, 404.

[8] Angier, R. B., *et al.*, *Science*, 1945, **102**, 227.

TABLE I

Composition of Basal Chick Diet.

	g/100 g		mg/100 g
Sucrose	58.0	Thiamine	0.30
Casein S.M.A. (vitamin-free)	18.0	Riboflavin	0.40
Gelatin	10.0	Pyridoxine	0.40
Salts (O & M)	5.0	d(+) Ca pantothenate	3.30
$CaHPO_4 \cdot 2H_2O$	1.0	Nicotinic acid	10.00
$MnSO_4 \cdot 2H_2O$	0.04	Mixed tocopherols	1.25
Corn oil	5.0	2-Methyl-1,4-naphthoquinone	0.44
Haliver oil	0.03	Biotin	0.036
Cystine	0.3	$ZnCl_2$	1.25
Choline	0.15	$CuSO_4 \cdot 5H_2O$	1.50
Inositol	0.1		

pure synthetic substance were used.* Liver concentrates used in these experiments were first standardized against a liver fraction of known vitamin B_c content.† The standardized liver fraction was later assayed against the synthetic *L. casei* factor. Standardizations against pure vitamin B_c and synthetic *L. casei* factor gave the same values with both *L. casei* and *S. faecalis R.* The active material in our concentrates is hereafter referred to as *L. casei* factor.

The basal diet was designed to supply in adequate amount all dietary factors which had previously been associated with chromotrichial activity in various species, excepting only *p*-aminobenzoic acid. Particular regard was paid to the adequacy of copper,[9,10,11] zinc,[12] pantothenic acid,[13,14,15] biotin,[16] and cystine.[17] The chromotrichial activity originally reported for *p*-aminobenzoic[18] acid in rats has not been verified.[19,15,2] For this reason it was decided not to include it in the diet at the present stage of investigation.

Experimental. Day-old Black Leghorn chicks were fed a basal diet as shown in Table I. In preliminary experiments, concentrates of the *L. casei* factor from liver were used to determine the levels needed for growth. Groups of 10 chicks were used and observations were recorded weekly. It was determined that a level of 400 μg per 100 g of diet gave good growth, feathering, and pigmentation over a period of 12 weeks. Growth was about 10% greater for control chicks receiving 10% liver fraction B (Wilson), but there was no observed difference in feathering and pigmentation. Chicks which received the *L. casei* factor concentrate for only the first 2 weeks grew at a decreasing rate and finally began to lose weight after 7 weeks; however, no failure in pigmentation or feathering was observed in this group even after 10 weeks.

Preliminary experiments were also conducted in which *L. casei* factor concentrates from liver were given 5 times weekly by injection in amounts calculated to supply 1, 2.5, 5, 10, and 20 μg of the factor per day. In this run the fullness of feathering was proportional to the level of *L. casei* factor up to the 10 μg

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* We wish to thank Dr. E. L. R. Stokstad of Lederle Laboratories for supplying us the synthetic *L. casei* factor used in these studies.

† This liver fraction was standardized against crystalline vitamin B_c by Prof. W. H. Peterson at the University of Wisconsin.

[9] Gorter, F. J., *Nature*, 1935, **136**, 185.

[10] Keil, H. L., and Nelson, V. E., *J. Biol. Chem.*, 1931, **93**, 49.

[11] Free, A. H., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 371.

[12] Stirn, E. E., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 347.

[13] György, P., and Poling, C. E., *Science*, 1940, **92**, 202.

[14] Unna, K., Richards, G. V., and Sampson, W. L., *J. Nutrition*, 1941, **22**, 553.

[15] Henderson, L. M., McIntire, J. M., Waisman, H. A., and Elvehjem, C. A., *J. Nutrition*, 1942, **23**, 47.

[16] György, P., and Poling, C. E., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 773.

[17] Pavcek, P., and Baum, H. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 271.

[18] Ansbacher, S., *Science*, 1941, **93**, 164.

[19] Emerson, G., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 448.

TABLE II.
Growth, Feathering, and Pigmentation of Chicks Receiving Various Levels of Synthetic *L. casei* Factor by Injection. Comparison with *L. casei* Factor Concentrate from Liver and with 10% Brewer's Yeast Supplement.

	Synthetic *L. casei* factor 1 μg			Synthetic *L. casei* factor 2.5 μg			Synthetic *L. casei* factor 5 μg		
Weeks	Wt, g	Feathering	Pigmentation %	Wt, g	Feathering	Pigmentation %	Wt, g	Feathering	Pigmentation %
1	44			46			46		
2	75			80			84		
3	106			127			146		
4	119			164			211		
5	132	Very poor	0	193	Poor	10	269	Subnormal	25
6	153	" "	0	234	"	10	310	"	40
7	157	" "	0	242	"	40	369	"	40
8	176	" "	0	291	"	40	436	"	60
9	172*	" "	10	308†	"	50	459	"	60

	Synthetic *L. casei* factor 10 μg			*L. casei* factor concentrate 10 μg			10% brewer's yeast		
Weeks	Wt, g	Feathering	Pigmentation %	Wt, g	Feathering	Pigmentation %	Wt, g	Feathering	Pigmentation %
1	44			45			45		
2	78			80			81		
3	130			135			147		
4	195			207			230		
5	264	Normal	100	275	Normal	90	300	Normal	100
6	346	"	100	348	"	90	358	"	100
7	374	"	100	409	"	90	458	"	100
8	477	"	100	495	"	90	594	"	100
9	548	"	100	544	"	90	666	"	100

* Only 3 chicks survived.
† Only 4 chicks survived.

level where it was normal. Depigmentation was marked at the 5 and 10 μg levels. At the lower levels, the feathers were poorly developed and the chicks had a ragged and scrawny appearance. Growth was in proportion to the level of *L. casei* factor given.

When synthetic *L. casei* factor was made available to us for research purposes, the above experiment was repeated at injection levels calculated to supply 1, 2.5, 5, and 10 μg daily of the crystalline material. A control group with 10% brewers' yeast supplement added to the basal diet was run for comparison. The results as to growth, feathering, and pigmentation were essentially similar to those above except that the 10 μg level of synthetic *L. casei* factor appeared adequate for normal pigmentation, whereas the concentrate at this level had not proved adequate.

The results with synthetic *L. casei* factor are shown in Table II. Photographs of representative chicks from the various groups are shown in Fig. 1.

At the 1 and 2.5 μg levels, feathering was so poor that true depigmentation was difficult to discern, although the chicks gave the general appearance of grayness. At the 5 μg level, chicks showed the most definite depigmentation. The plumage of this group was somewhat sparse, but was full enough so that depigmentation was readily apparent. Both feathering and pigmentation were about equal for the group receiving 10 μg *L. casei* factor by injection and the control group fed yeast supplement.

Depigmentation became most pronounced in the proximal portions after the feathers were fairly well developed. The wing and tail feathers were most affected, but nearly all of the feathers were somewhat affected. Depigmentation was not discernible as quickly in the lower level groups as it was at the 5 μg level, probably because feather growth was

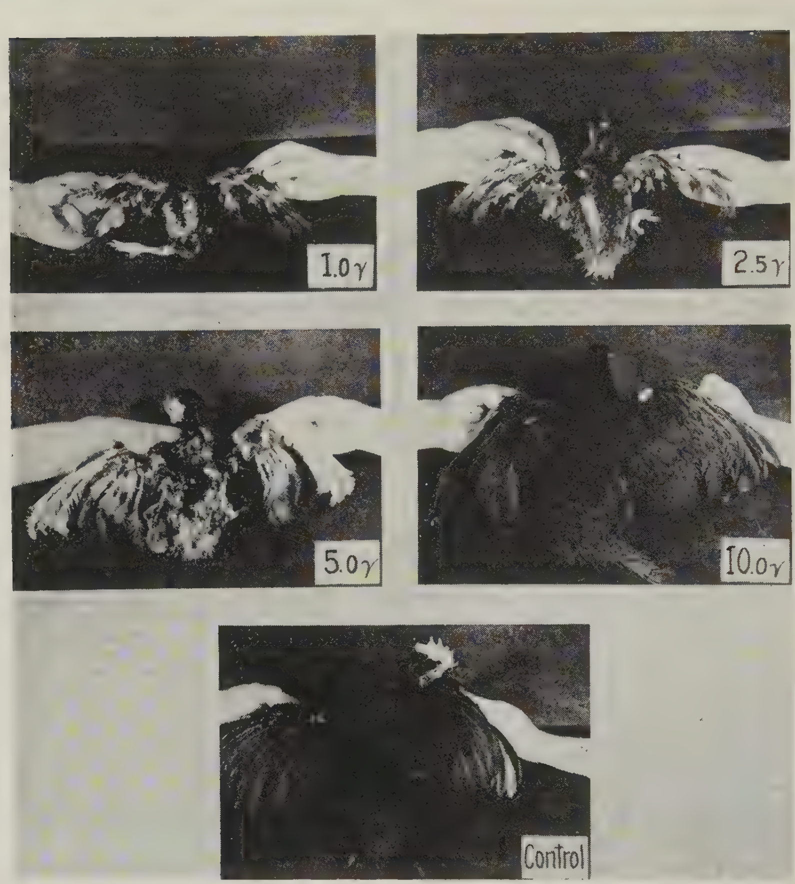

FIG. 1.
Chicks at nine weeks of age representing the groups which received 1, 2.5, 5, or 10 μg daily of synthetic *L. casei* factor by injection or 10% brewer's yeast added to the basal diet.

slower and less complete at the lower levels.

Discussion. A primary need for the *L. casei* factor in the chick would appear to be for feather development. When the need for feather formation is just satisfied, the needs for pigmentation are still not met. Depigmentation then becomes most apparent in the rapidly growing feathers. A still higher level is needed to satisfy both needs completely.

Campbell, Brown, and Emmett[20] have indicated that a level of 20 μg daily of crystalline vitamin B_c isolated from yeast, given either orally or parenterally to growing chicks, produces normal growth, feathering, and blood values. The present work indicates that a level of about 10 μg of synthetic *L. casei* factor is adequate for normal feathering and gives good growth. It appears likely that a somewhat higher level would have given growth comparable to that obtained with 10% brewers' yeast supplement. Evidence for the existence of separate factors required for feathering and growth has been presented by Briggs, Luckey, Elvehjem, and Hart.[21] The multiplicity of forms in which the *L. casei* factor apparently occurs in nature requires clarification with regard to this possibility.

In separate experiments we have used concentrates of a fermentation product highly active for *S. faecalis R.*, but only slightly active for *L. casei.* These concentrates gave very poor growth and feathering when used at a level which proved adequate in the case of liver concentrates, based on *S. faecalis R.* assay. Thus it may prove true that the *L. casei* assay is a better indication of activity for higher animals.

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[20] Campbell, C. J., Brown, R. A., and Emmett, A. D., *J. Biol. Chem.*, 1944, **154**, 721.

[21] Briggs, G. M., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1944, **153**, 423.

The importance of pantothenic acid in normal pigmentation has been adequately emphasized. The interesting observation has been further made[6] that the storage and utilization of pantothenic acid in succinylsulfathiazole-fed rats is favorably influenced by the simultaneous administration of biotin and folic acid-rich concentrates. Early work in this laboratory[1] indicated that low levels, *i.e.*, 40-60 μg of pantothenic acid as it occurred in natural products, were much more effective in prevention of graying in growing rats than levels up to 200 μg per day of d(+)calcium pantothenate. The inference was that other factors were acting synergistically with pantothenic acid. This idea seems to have support in much of the work to date, and it appears probable that the metabolic functions of the *L. casei* factor and pantothenic acid are closely interdependent.

In line with the above reasoning it may now become possible to reconcile the discrepancies between different laboratories regarding the adequacy of pantothenate alone to cure or prevent achromotrichia in rats. The adequacy of given levels of pantothenate for growth and pigmentation may to some extent be governed by the level of *L. casei* factor in the diet, or the rate of intestinal synthesis of this factor. Unna and Richards[22] observed that the requirement of pantothenate for rats decreases from 100 μg per day at 3 weeks of age to 25 μg per day at 10 weeks of age. Furthermore, rats started on 25-50 μg per day of pantothenate became gray but showed gradual restoration of the black color of the fur as they approached maturity. These observations were confirmed by Henderson *et al.*[15] On the other hand, Williams[23] reported the inefficacy of even high levels of pantothenate to cure graying uniformly, as was also observed in this laboratory.[1] Further experiments are indicated in which the *L. casei* factor is supplied together with pantothenate.

The needs of the chick for the *L. casei* factor are apparently not complicated by intestinal synthesis to the same extent as in the rat. For the latter reason, chicks may be considered preferable to other species in studies dealing with the effect of diet on normal pigmentation processes.

It is significant to note that Willier[24] has elaborated on the common role of melanophores in bringing about the pigmentation of hair or feathers in different species and has further postulated that a genetic lethal factor in the melanophore may be the primary cause of graying in man.

Summary. Black Leghorn chicks were raised on a basal diet designed to contain the previously recognized chromotrichial factors, except the *L. casei* factor. Synthetic *L. casei* factor injected 5 times weekly at a level calculated to supply 10 μg per day showed feathering and pigmentation comparable to that of chicks receiving 10% brewers' yeast supplement. Growth was somewhat better on the latter. At a level of 5 μg, feathering was fairly good and depigmentation was marked. At 1 and 2.5 μg levels, feathering was very poor. Growth and feathering were in proportion to the level of *L. casei* factor used.

Acknowledgment is made to Mr. Albin Junnila for technical care of the animals.

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[22] Unna, K., and Richards, G. V., *J. Nutrition*, 1942, **23**, 545.

[23] Williams, R. R., *Science*, 1940, **92**, 561.

[24] Willier, B. H., *Ann. Surgery*, 1942, **116**, 598.

15228

Effect of Radon-Containing Ointment on Cutaneous Lymph Flow and Minute Vessels of Skin.

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From the Robinette Foundation and the Department of Radiology, Hospital of the University of Pennsylvania.

It had been the clinical experience of one of us (E.P.P.) that "Alphatron" Radon Ointment† was of value in the treatment of ulcers resulting from previous excessive irradiation. The study here reported is an attempt to explain this effect. For clarity of presentation, the evidence will be presented in the following order: (1) Experiments suggesting that decreased lymphatic absorption, and presumably therefore some degree of lymphatic block, is present following the subsidence of the acute stage of post-irradiation reaction. (2) Experiments indicating that there is hyperemia of the active type in man and in animals following the use of Radon ointment. (3) Experiments showing in man and in normal animals there is increased lymphatic flow in the cutaneous area treated with Radon ointment. (4) Experiments in animals indicating that the active hyperemia and increased cutaneous lymphatic flow in animals is not associated with increased permeability of the capillaries to the colloidal dye Congo red, whereas such increase in permeability does occur following excessive irradiation by x-rays. (5) Result of the application of Radon ointment to cutaneous areas in men in whom local lymphatic block was presumed to have occurred following the injection of penicillin.

The normal human subjects were men between the ages of 22 and 41; the abnormal subjects, who received the penicillin, were men between the ages of 30 and 50 who were being treated for subacute bacterial endocarditis. The animals used were white rats, of either sex, some normal and others, where stated, having been previously treated with roentgen irradiation totaling 2385 r, given in a single dose. Radon ointment in all cases contained 500 electrostatic units per cubic centimeter, was applied in a film approximately 1 mm thick, covered with oil silk fastened down at the edges by adhesive tape, and left in place for 4 hours when it was removed by gentle wiping with cotton. Control areas were treated similarly but using anhydrous lanolin instead of Radon. Studies were made 24 hours after the application of the Radon ointment, unless otherwise expressly stated.

1. Lymphatic absorption following x-ray irradiation: Lymphatic flow in the skin may be measured either by (a) injecting a colored colloidal dye as patent blue[1] into the skin and measuring the speed and extent of streamer formation, or (b) measuring the amount of time required for complete absorption of a standard amount of dye so injected. The first method, measuring streamers, is well adapted to show increased lymphatic flow, but normal flow is so slow that decreased flow cannot be so demonstrated; the second method is better adapted to demonstrating diminished flow.

Sixteen rats were used that 3 months previously had received roentgen therapy to one leg. Patent blue, 0.04 cc in amount, was injected intracutaneously into the dorsum of both feet, one irradiated, and the other a control. After 24 hours, the dye had completely disappeared from both feet in 6 rats, while in the other 10 it was present on the irradiated but not on the control side.

2. Hyperemia following application of Radon ointment: The antecubital area of the forearm was examined under the capillary

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* Atwater Kent Fellow in Medicine.

† Obtained from the Canadian Radium and Uranium Corporation, 630 Fifth Ave., New York City, which also provided funds to defray part of the expense of the study.

[1] McMaster, P. D., *J. Exp. Med.*, 1937, **65**, 347.

microscope[2] before and 24 hours after the application of Radon ointment, and compared with the other arm prepared as a control. The appearance of the control arm was the same on both days. The treated arm showed an increase in capillary loops of approximately 100% in 7 subjects, along with some increase in subpapillary venous plexus in 5 of the subjects. The color was bright red, not cyanotic, but otherwise it could not be told whether the hyperemia was active or passive. However, the dorsum of the foot of 6 rats, similarly treated and with the opposite leg as a control, indicated quite clearly that the hyperemia was active. In this area, the rat does not open and close cutaneous vessels as does man in the forearm, but usually only about 30% of the vessels show flow. This was true of the control leg on both days and of the treated leg before treatment, but after treatment about 90% of the vessels showed flow, a result very comparable to that obtained 4 or 5 days after lumbar sympathectomy.

3. Lymphatic flow following application of Radon ointment: Radon ointment was applied in the usual way for 4 hours to the antecubital area of one arm in 8 normal men, and 12 hours later 0.04 cc of patent blue was injected intracutaneously into the treated area and into a corresponding area of the control arm. Streamer formation was followed at 5 minute intervals for 15 minutes, and found to be significantly increased on the treated side in 6 cases and not significantly different in 2 cases. Disappearance time was measured in 5 cases, and ranged from 8 to 10 hours, averaging 9 hours, on the treated side, and 24 to 48 hours, averaging 36 hours, on the control side.

Under sodium phenobarbital anesthesia lasting about 24 hours, 14 rats were treated with Radon ointment to one foot using the same dosage and time factors as for the human subjects. However, immediately after the Radon and control dressings were removed, both feet were injected intracutaneously with an equal amount of patent blue. Twenty hours later it was noted that dye was present in the control foot but not in the treated foot of 7 animals, and had cleared entirely from both feet in the remaining 7 animals. In no case was dye present in the treated leg after the control leg had cleared.

4. Permeability of capillaries to Congo red after the application of Radon ointment: Under sodium phenobarbital anesthesia, Radon ointment was applied to one foot of 8 normal rats, and immediately thereafter 0.3 cc of a 5% solution of the colloidal dye Congo red was injected into a jugular vein. The animals were observed at intervals for 24 hours. At no time was the treated leg any pinker than the control leg or the rest of the body. By contrast, Congo red was injected similarly into 7 animals that had received 2385 r of x-ray irradiation to one leg one month previously. Within 5 minutes the treated leg had become very red, as compared with the control leg and other parts of the body.

5. Four men were available in the medical ward who had received many injections of penicillin. One man in particular had received hundreds of injections over a period of 8 months, with so much resulting discomfort that he was about to refuse further therapy. It seemed likely that the local swollen areas represented areas of local lymphedema due to acute partial lymphatic block from colloidal and particulate matter in the penicillin. Improvement was striking in 12 hours. An arm that had been held rigidly because of a painful swelling near the scapula was freely moveable and the swelling was greatly decreased in size and not tender. Previous therapy, over a period of more than 3 months, with heat, baking, light et cetera had been completely ineffective in relieving the pain and swelling. In all, 12 applications of Radon ointment were given to 4 patients over areas where a painful swelling had appeared following an injection of penicillin. Six control applications were given using either the inactive ointment or Radon ointment from a tube at least 2 weeks old. In every case the control applications were recognized by the patient as being ineffective, and the treatments as effective.

Summary and Conclusions: Evidence is

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[2] Griffith, J. Q., Jr., Roberts, E., and Corbit, H. O., *Am. Heart J.*, 1941, **21**, 47.

presented suggesting that the effect of Radon ointment applied to the skin of men and animals is: (1) To produce active hyperemia. (2) To increase lymph formation and lymph flow without appreciably increasing capillary permeability to colloids. (3) It is suggested that the therapeutic effect of Radon ointment may be apparent in these cases where lymph flow is diminished, perhaps due to partial block. Evidence is offered suggesting that lymph flow is diminished in a limb that has, in the past, been exposed to excessive x-ray irradiation. Also, the result of treating acute lesions thought to represent partial lymphatic block following penicillin injections is described, with results that are suggestively good although the series is too small for absolute certainty.

15229

Effects of Glutathione and Thioglycollic Acid on Acid-Production by *Lactobacillus arabinosus* in Presence of Atabrine.

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(Introduced by M. Pittman.)
From the National Institute of Health, Bethesda, Md.

The action of atabrine as a competitive inhibitor of certain enzyme systems has been investigated by Wright and Sabine[1] and Haas.[2] The influence of different types of diets upon the toxicity of atabrine for various laboratory animals has been studied by several groups of workers, and a recent article by Siegel, Mushett and Emerson[3] reviews the contributions in this field.

Silverman and Evans[4] reported on the effects of spermine, spermidine, and other polyamines on the growth inhibition of *Escherichia coli* and certain soil organisms by atabrine. These organisms are capable of growth on an inorganic salts-glucose medium and atabrine bacteriostasis is relieved by spermidine. On the other hand, they observed that atabrine bacteriostasis produced in soil organisms with complex nutrient requirements is not relieved by spermidine and related polyamines.

The purpose of this paper is to present data respecting the response of the *Lactobacillus arabinosus* (an organism with relatively complex nutrient requirements) to atabrine with and without the addition of glutathione and thioglycollic acid to the medium.

Materials and Methods: A strain of *Lactobacillus arabinosus* 17-5 was used. This was supplied by Dr. Jerald G. Wooley of the Division of Physiology, National Institute of Health. The procedure for growth of stock cultures and preparation of culture for inoculation was that of the U. S. Pharmacopeia,[5] using the technics employed by the Food and Drug Administration. The basal medium and stock solutions were that of the U. S. Pharmacopeia for nicotinic acid assays[5] except that 0.25 g of bacto-dextrose, 0.20 g of sodium acetate and 0.5 μg of nicotinic acid were added to each tube, *i.e.*, 10 ml final volume.

A distilled water solution of C.P. atabrine dihydrochloride dihydrate (2.0×10^{-3}M) was added to the tubes in varying amounts in such a range that minimum and maximum bacteriostatic effects were demonstrated. Comparable tubes were set up which contained distilled water solutions of glutathione, Merck, or thioglycollic acid practical, Eastman, in a final concentration of 1.6×10^{-3} molar. In these experiments the pH of the medium was not altered by the addition of

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[1] Wright, C. I., and Sabine, J. C., *J. Biol. Chem.*, 1944, **155**, 315.

[2] Haas, E., *J. Biol. Chem.*, 1944, **155**, 321.

[3] Siegel, H., Mushett, C. W., and Emerson, G. A., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 157.

[4] Silverman, M., and Evans, E. A., *J. Biol. Chem.*, 1944, **154**, 521.

[5] *U. S. Pharm.*, XII First Bound Supplemeent, 1943.

TABLE I.
Percent Inhibition of Acid Production by *Lactobacillus arabinosus* in the Presence of Atabrine Alone, Atabrine + Glutathione, and Atabrine + Thioglycollic Acid.

Molar concentration of atabrine in tube	% inhibition of acid production Atabrine alone %	Atabrine + glutathione %	Atabrine + thioglycollic acid %
0	0	0	0
1.0×10^{-4}	24	27	6
1.2×10^{-4}	42	28	14
1.6×10^{-4}	91	50	29
2.0×10^{-4}	100	85	56
2.4×10^{-4}	98	98	90

glutathione and thioglycollic acid. The amount of bacterial growth after autoclaving, inoculation and incubation for 66 hours was measured by titrating each tube for acid production with 0.1 N NaOH. A blank was always run with all the ingredients but no inoculation; the amount of 0.1 N NaOH required for this blank was routinely subtracted from the titration values of the comparable inoculated tube. One hundred percent growth was the value obtained in the control tube which contained no atabrine and was inoculated. The percent inhibition of growth induced by atabrine was computed by comparing the acid production by titration under control conditions, when no atabrine was added, with acid production under experimental conditions when atabrine was added. When the effect of glutathione and thioglycollic acid was being tested the control tubes without atabrine contained an amount of glutathione or thioglycollic acid comparable to that put in the experimental tubes.

Results. The percent inhibition of acid production with atabrine alone, atabrine plus glutathione, and atabrine plus thioglycollic acid is shown in Table I. The data demonstrate that glutathione and thioglycollic acid are able to reverse the bacteriostatic effect of atabrine over a restricted range of concentration of the latter. For example, when the molar concentration of atabrine was 1.6×10^{-4} the percent inhibition of acid production was 91%, but with a similar concentration of atabrine plus glutathione the inhibition was 50%, and only 29% in the case of atabrine plus thioglycollic acid. In the experiments carried out it was not possible to counteract the effects of the higher molar concentrations of atabrine by increasing the amounts of glutathione and thioglycollic acid. Lower concentrations of glutathione and thioglycollic acid than 1.6×10^{-3} were effective in counteracting atabrine but the results were not consistent.

In addition to the above compounds, which counteracted the effect of atabrine, several others were tried under comparable conditions with negative results; these included: d-cystine, l-cystine, cysteine, dl-methionine, choline, nicotinic acid, riboflavin, and pyridoxine.

In the case of *E. coli* it has been shown[6,4] that shifting the pH of the medium to values below 6.0 (which permit satisfactory growth of *E. coli*) is effective in reversing the action of atabrine. This was tried in the case of *Lactobacillus arabinosus* and a comparable effect was attained.

Summary. Under the conditions of these experiments glutathione and thioglycollic acid are capable of reversing the growth inhibiting effect of atabrine on the *Lactobacillus arabinosus*. Once the concentration of atabrine for complete inhibition had been significantly exceeded, no reversal of bacteriostasis was attained by increased concentration of glutathione and thioglycollic acid. Several additional compounds were tested under comparable conditions with negative results.

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[6] Browning, C. E., Gulbransen, R., and Keenaway, E. L., *J. Path. and Bact.*, 1919, **23**, 106.

15230 P

Lactation Leucopenia in Rats on Purified Diets.*

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Recently long-term experiments have shown that purified diets for rats are equivalent to stock diets for growth[1,2] and reproduction[2] but not for lactation.[1,2,3] The criteria used for lactation have been the percentage of young weaned, their average weight at weaning, and the loss in weight of the lactating mother during the lactation period. In comparison with the 60-70% weaning reported by other investigators[1,3] working with purified diets, in this laboratory rats maintained throughout their entire life on a purified diet (836‡) have weaned 90% of their young.[2] Since this is equivalent to the lactation performance of stock animals in our rat colony, this criterion has been eliminated from our work. By placing adult stock animals with their litters on the purified diet at parturition it is possible to test dietary factors in 3 weeks for their effects on the weaning weight of the young and the weight loss of the mother.[2] We now wish to report an additional criterion for testing the adequacy of purified diets, namely, the occurrence of leucopenia in the lactating mother during this short period.

Procedure. Adult stock females with their litters were placed on the purified diet (836‡) at parturition or were continued on the stock diet[5] as controls. The litters were limited to 6 young, preferably 3 males and 3 females, which were weaned on the twenty-first day following parturition. Total and differential white blood counts were carried out by standard procedures at 2 different times, near the beginning of the lactation period (Day 5) and at the time of weaning (Day 21).

Results and Discussion. Table I shows typical results for rats maintained on the stock diet or on the purified diet with or without supplements. There was no change in total WBC, lymphocytes and monocytes, or granulocytes during the lactation period in animals maintained on the stock diet whereas a marked leucopenia occurred by the twenty-first day in animals maintained on the purified diet (836). All animals receiving the purified diet (without any supplementation) showed a significant decrease in granular and non-granular leucocytes from their original level (Day 5) and the drop to pathological levels of granulocytes (*i.e.* granulocytopenia, less than 750 cells per

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† General Mills Fellow.

[1] Vinson, L. J., and Cerecedo, L. R., *Federation Proc.*, 1943, **2**, 73; *Arch. Biochem.*, 1944, **3**, 389.

[2] Nelson, M. M., and Evans, H. M., in preparation.

[3] Richardson, L. R., and Hogan, A. G., *Federation Proc.*, 1945, **4**, 161.

[4] Salts No. 4 of Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1944, **138**, 459.

‡ Basal diet 836 consists of alcohol-extracted casein 24%, sucrose 64%, hydrogenated vegetable oil (Crisco) 8%, and salts[4] 4%. Crystalline B vitamins are added per kilogram diet: thiamine HCl 5 mg, pyridoxine HCl 5 mg, riboflavin 10 mg, p-aminobenzoic acid 10 mg, nicotinic acid 20 mg, calcium pantothenate 50 mg, inositol 400 mg, and choline chloride 1 g. One cc of a fat-soluble vitamin mixture containing 6 mg alpha-tocopherol, 115 chick units vitamin D, 800 U.S.P. units vitamin A, and 650 mg corn oil (Mazola) is given weekly to each litter.

[5] Wainwright, W. W., and Nelson, M. M., *Am. J. Orthodontics and Oral Surgery*, 1945, **31**, 406.

TABLE I.
Maternal Leucocytes During Lactation.

Experimental diet	Day of lactation (No. of rats)	Total* WBC	Lymphocytes and monocytes %	Lymphocytes and monocytes Total*	PMN %	PMN Total*
Stock Diet I + lettuce	Day 5 (10)	13,095 (8,000-21,000)	70%	9,325 (5,200-14,175)	30%	3,770 (1,710-4,945)
	" 21 (10)	12,375 (7,350-17,300)	72%	9,035 (4,770-14,530)	28%	3,340 (2,350-4,720)
Purified Diet 836	" 5 (7)	11,845 (6,150-17,400)	72%	8,430 (4,670-10,265)	28%	3,415 (1,480-7,135)
	" 21 (11)	5,205 (2,625-9,600)	83%	4,195 (2,310-7,200)	17%	1,010 (215-2,730)
Purified Diet 836 + 1 g brewers' yeast daily	" 5 (8)	12,850 (8,500-19,000)	69%	9,025 (4,385-16,500)	31%	3,825 (1,700-6,690)
	" 21 (9)	7,200 (3,400-12,350)	79%	5,530 (3,400-10,375)	21%	1,670 (0-4,405)
Purified Diet 836 + 1 cc liver extr. daily	" 5 (8)	13,245 (10,800-17,000)	68%	9,000 (6,780-13,040)	32%	4,245 (1,860-7,515)
	" 21 (8)	8,005 (5,670-9,775)	74%	5,950 (3,995-8,435)	26%	2,055 (645-3,400)

* The range is given in parentheses below the average value.

mm^3) occurred in two-thirds of the group. Daily oral supplementation with 1 cc liver extract (Lederle 93% alcohol liver concentrate H-9381-B) resulted in some improvement but not in a return to the normal blood picture; 1 g brewers' yeast was somewhat less beneficial.

Preliminary studies were carried out with a folic acid concentrate (potency 5,000) kindly furnished by Dr. R. J. Williams, University of Texas. Oral supplements of this concentrate, equivalent to 150 μg "folic acid" (*i.e.* 7.5 μg daily) were given during the 3-week period to the lactating mothers without beneficial effects on any of the criteria for lactation. While the effects of "folic acid" concentrates on the other criteria for lactation are questionable,[3,6] there is no doubt concerning the curative effects of such concentrates or of crystalline vitamin Bc (total dose 100 μg) on the leucopenia and granulocytopenia of non-lactating rats maintained on purified diets with sulfa drugs[7] or even without their addition.[8] Either considerably higher levels of "folic acid" or additional factors must be necessary for a normal leucocyte picture during the strain of lactation.

Summary. Adult stock rats maintained on stock diets show no changes in the white blood cells during the 3-week lactation period. Maintenance on a purified diet during the same period results in a marked leucopenia and granulocytopenia by the twenty-first day. Daily supplements of brewers' yeast or liver extract result in improvement but not entire prevention of the blood dyscrasia.

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[6] Cerecedo, L. R., and Vinson, L. J., *Arch. Biochem.*, 1944, **5**, 469.

[7] Sebrell, W. H., and Daft, F. S., *Pub. Health Rep.*, 1943, **58**, 1542.

[8] Kornberg, A., Daft, F. S., and Sebrell, W. H., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 46.

15231

Identification of Rickettsial Agents Isolated in Guinea Pigs by Means of Specific Complement Fixation.

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The isolation of the agents of rickettsial diseases of man are usually made in guinea pigs (scrub typhus is an exception, since the white mouse is more susceptible). Once isolated the agent is identified by its capacity to induce a febrile and pathologic response in guinea pigs (scrotal swelling or necrosis, enlarged spleen, etc.) or the absence of these reactions in other species of animals, followed by the demonstration of a reciprocal cross immunity with known rickettsial strains. This procedure is not only time-consuming and expensive but presents certain difficulties because strains of the agent of a given disease may induce reactions in guinea pigs of different degree and intensity. The problem of strain identification, based on animal reactions alone, can therefore, present certain difficulties, some of which are discussed here.

When human blood, from a case of epidemic typhus, is inoculated intraperitoneally into male guinea pigs (400-500 g), the incubation period may vary from 6 to 20 days followed by a febrile period of variable duration. When brain suspensions from these animals are passaged to other male guinea pigs the incubation period is shortened to 6 or 7 days but may vary from 4 to 14 days. (Data based on 54 separate isolations).†

While scrotal swelling is not frequent in epidemic typhus, this reaction may occasionally occur. Since epidemic and murine typhus confer a reciprocal cross immunity in guinea pigs further identification of the agent is made in white rats, in which animal the epidemic disease cannot be maintained by serial passage, while this is possible with murine typhus. Since fever is an important expression of the experimental disease, the interpretation of a reaction can be confused when fever is caused by conditions other than typhus. Furthermore, there are a certain number of guinea pigs that develop an inapparent disease, which is characterized by the absence of a febrile reaction.

When infectious human blood from a case of murine typhus is inoculated intraperitoneally into male guinea pigs (400-500 g), the incubation period can vary from 4 to 14 days followed by a febrile period of variable duration. When scrotal exudate from these animals is passaged to other guinea pigs, the incubation period is usually shortened to about 4 or 5 days with a variation of from 2 to 10 days. This is then followed by a febrile period of variable duration.† While scrotal swelling is usually present in large guinea pigs and Neil-Mooser cells are readily demonstrated, this reaction may be absent in animals even though they develop a febrile reaction. There are other guinea pigs that develop an inapparent disease, with no febrile or scrotal reaction whatsoever.

The type of reaction induced in guinea pigs with the agent of Rocky Mountain spotted fever depends upon whether a mild or virulent strain is isolated. The period of incubation with the former is usually longer; furthermore, no scrotal necrosis occurs and the guinea pigs usually survive. With the virulent strain, the period of incubation is shorter, scrotal necrosis develops and death occurs in the majority of the animals inoculated. Identification of the spotted fever agent by cross immunity tests may cause some difficulty since a partial immunity to Rocky Mountain spotted fever is found in guinea pigs convalescent from typhus

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* Member, United States of America Typhus Commission.

† Same technic used throughout. Details to be published.

TABLE I.
Murine Typhus.

Guinea pig No.	Incubation (days)	Fever (days)	Scrotal reaction	Complement fixation titer: Epidemic	Complement fixation titer: Murine	Complement fixation titer: Rocky Mountain spotted fever
3322	3	7	+	1/10	1/320	0
3328	3	7	+	1/20	1/160	0
3329	3	7	+	1/20	1/320	0
3325	4	6	+	1/10	1/160	0
3326	3	6	+	1/20	1/320	0
3330	3	6	0	1/20	1/320	0
3338	3	6	+	1/80	1/320	0
3347	4	6	0	1/80	1/640	0
3317	3	5	+	1/80	1/320	0
3319	3	5	+	0	1/160	0
3321	3	5	+	1/20	1/160	0
3331	3	5	+	1/10	1/160	0
3332	3	5	+	1/10	1/160	0
3334	3	5	+	1/20	1/320	0
3337	3	5	+	1/10	1/80	0
3341	4	5	0	1/40	1/320	0
3323	4	4	+	1/20	1/320	0
3335	5	4	+	1/80	1/640	0
3344	5	4	+	1/20	1/160	0
3350	3	4	+	1/10	1/160	0
3308	6	3	0	1/40	1/320	0
3311	3	3	0	1/10	1/320	0
3316	5	3	+	1/80	1/320	0
3318	4	3	+	0	1/160	0
3320	4	3	+	1/20	1/320	0
3324	4	3	+	1/20	1/320	0
3327	5	3	+	1/20	1/320	0
3349	5	3	+	0	1/160	0
3355	3	3	+	1/40	1/320	0
3307	5	2	0	1/10	1/160	0
3309	5	2	0	1/40	1/320	0
3313	4	2	+	1/40	1/640	0
3333	7	2	0	1/20	1/320	0
3339	4	2	0	1/20	1/320	0
3345	5	2	+	1/20	1/320	0
3352	5	2	+	1/80	1/640	0
3306	5	1	+	1/20	1/160	0
3314	6	1	+	1/40	1/320	0
3346	5	1	+	1/20	1/320	0
3354	5	1	+	1/20	1/320	0
3342	0	0	0	1/20	1/320	0
3348	0	0	0	1/10	1/160	0

fever and vice versa.[1,2]

During the past 3 years, this laboratory has employed the specific complement fixation test on specimens of convalescent guinea pig serum to differentiate epidemic and murine typhus and Rocky Mountain spotted fever. This paper will deal with the results obtained in an experimental study of infections in guinea pigs produced with strains of these 3 agents.

Experimental. Murine Typhus: A series of 50 male guinea pigs (400-500 g) was bled prior to inoculation in order to obtain baseline specimens. They were then inoculated intraperitoneally at the same time with the same pooled suspension of 1 cc of tunica vaginalis exudate—Wilmington strain. (Both tunica vaginalis and testicular exudate are suspended in 20 cc of physiological saline.) Forty-two of the 50 guinea pigs survived the entire experiment, the others dying of intercurrent infections.

Forty guinea pigs developed fever while 2

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[1] Castaneda, M. R., and Silva, R., *J. Immunol.*, 1941, **42**, 1.

[2] Parker, R. R., *Public Health Rep.*, 1943, **58**, 721.

showed no febrile reaction. The period of incubation varied from 3 to 7 days and the febrile period from 1 to 7 days. Eleven or 26% of the 42 guinea pigs did not develop a scrotal reaction and 2 of these likewise did not develop fever.

About 4 weeks after the initial inoculation, all of the guinea pigs were bled and complement fixation tests for epidemic and murine typhus and Rocky Mountain spotted fever were performed, using the specific purified rickettsial antigens already described.[3,4,5,6] It had been shown that the soluble or "common" antigen gave cross fixation between epidemic and murine typhus and, hence, differentiation between these diseases could not be made when this material was present. Differentiation between these closely related diseases was possible, however, when washed rickettsial suspensions, free of soluble antigen, were used as antigen. Table I summarizes the results obtained.

All pre-inoculation specimens were negative in complement fixation tests with the epidemic, murine and Rocky Mountain spotted fever antigens. The convalescent specimens all gave positive reactions with the murine antigen; the titers varied between 1/80 to 1/640 with values of 1/320 or greater in more than half of the specimens. All but 3 of the sera also reacted with the epidemic antigen; nevertheless, the titers were always considerably lower than those obtained with the murine antigen. The serological response of guinea pigs 3307, 3309, 3311, 3330, 3333, 3339, 3341, 3342, 3347 and 3348, which developed a febrile reaction without scrotal swelling, was indistinguishable from that of guinea pigs which developed both fever and scrotal swelling. Guinea pigs 3342 and 3348 were of particular interest, for they had neither a febrile reaction nor scrotal swelling, yet they also developed complement fixing antibodies. This serological response, together with the subsequent demonstration of an immunity to re-infection, indicated that these 2 guinea pigs had had an inapparent infection. There appears to be no correlation between the titers obtained and the severity of the experimental infection, as judged by the duration of the febrile period or the presence or absence of scrotal swelling. All specimens of serum were negative in tests with the Rocky Mountain spotted fever antigen.

One month after the temperatures returned to normal the guinea pigs were challenged by the intraperitoneal inoculation of 1 cc of a tunica vaginalis exudate suspension (Wilmington strain). All of the guinea pigs proved immune, while the controls reacted in the usual manner.

Epidemic Typhus. Fifty male guinea pigs (400-500 g) were bled for determination of pre-inoculation antibody levels and then injected intraperitoneally at the same time with 1 cc of a 10% pooled brain suspension (Breinl strain—guinea pigs sacrificed on the third febrile day). Thirty-nine of the 50 guinea pigs survived the entire experiment, the others dying of intercurrent infection. Thirty-three guinea pigs developed fever while 6 showed no febrile reaction. The period of incubation varied from 5 to 19 days followed by a febrile period of from 1 to 10 days. Four of the guinea pigs developed scrotal swelling. About 4 weeks after the initial inoculation all of the guinea pigs were bled and complement fixation tests for epidemic and murine typhus and Rocky Mountain spotted fever were performed on samples of serum. Table II summarizes the results obtained.

All pre-inoculation specimens gave negative results in complement fixation tests with the epidemic, murine or Rocky Mountain spotted fever antigens. Sera from 32 of the 33 guinea pigs that developed fever showed complement fixation titers of 1/80 to 1/640, the majority being 1/320 or higher, with the epidemic antigen. When cross fixation occurred with the murine antigen, the titers in these 8 instances were low, never exceeding 1/40. None of the specimens reacted with the Rocky

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[3] Plotz, H., and Wertman, K., *Science*, 1942, **95**, 441.

[4] Plotz, H., *Science*, 1943, **97**, 20.

[5] Plotz, H., Wertman, K., Bennett, B. L., Report to the Surgeon General, February 15, 1944, to be published.

[6] Plotz, H., Wertman, K., and Reagan, R. L., *Bulletin of the U. S. Army Medical Department*, 1944, **79**, 40.

TABLE II.
Epidemic Typhus.

Guinea pig No.	Incubation (days)	Fever (days)	Complement fixation titer		
			Epidemic	Murine	Rocky Mountain spotted fever
764	6	10	1/320	1/10	0
765	5	9	1/320	0	0
771	5	9	1/320	0	0
981	5	9	1/640	1/20	0
759	8	8*	1/160	0	0
761	6	8*	1/320	1/10	0
768	5	8	1/160	0	0
994	6	8	1/160	0	0
736	8	7	1/320	0	0
738	6	7*	1/640	1/40	0
758	7	7	1/320	0	0
767	9	7	1/160	0	0
769	5	7	1/320	0	0
740	6	6	1/160	0	0
775	7	6	1/640	0	0
979	6	6*	1/320	0	0
980	5	6	1/320	0	0
984	7	6	1/320	0	0
739	7	5	1/320	0	0
757	12	5	1/80	0	0
770	10	5	1/160	0	0
742	9	4	1/320	1/10	0
743	7	4	1/640	0	0
772	10	4	1/160	0	0
773	9	4	1/160	0	0
987	9	4	1/320	0	0
990	10	4	1/160	1/20	0
991	6	4	1/160	0	0
766	5	3	1/160	1/20	0
760	6	3	1/640	1/20	0
977	19	2	1/640	0	0
985	7	1	1/80	0	0
986	15	1	0	0	0
737	0	0	0	0	0
741	0	0	0	0	0
763	0	0	0	0	0
983	0	0	0	0	0
988	0	0	0	0	0
989	0	0	1/160	0	0

* Also had scrotal swelling.

Mountain spotted fever antigen. Guinea pig 986, that developed no complement fixing antibodies, had fever of 104.5° F. for one day's duration on the 15th day after inoculation. When subsequently challenged, with an epidemic strain, this animal responded with a febrile reaction; furthermore, the epidemic complement fixing antibodies then appeared in its serum. Thus the fever which was observed in this animal on the 15th day after primary inoculation was due to causes other than epidemic typhus. The other 31 members of the group were immune when challenged with epidemic typhus material.

There were 6 guinea pigs that did not develop a febrile reaction after the initial inoculation of infectious material (see No. 737, 741, 763, 983, 988, and 987 in Table II). It is observed that one of these guinea pigs, No. 989, developed specific epidemic complement fixing antibodies in convalescence. This animal proved to be immune to epidemic typhus when challenged subsequently. Therefore, guinea pig No. 989 had an inapparent infection as a result of the primary inoculation. In contrast, the other 5 guinea pigs in this group were not immune when challenged with an epidemic strain. It is considered

TABLE III.
Rocky Mountain Spotted Fever.

Guinea pig No.	Incubation (days)	Fever (days)	Complement fixation titer		
			Epidemic	Murine	Rocky Mountain spotted fever
294	3	9	0	0	1/320
280	3	8	0	0	1/320
323	2	8	0	0	1/320
290	3	7	0	0	1/640
292	4	7	0	0	1/320
276	3	7	0	0	1/640
260	4	7	0	0	1/320
286	4	7	0	0	1/320
288	4	7	0	0	1/320
190	6	7	0	0	1/320
191	2	6	0	0	1/320
284	5	5	0	0	1/320
189	6	2	0	0	1/640

that these animals represent "misses" in that the inoculated infectious material induced neither fever, an antibody response nor immunity.

In another series of 50 guinea pigs infected with different strains of epidemic typhus, 2 guinea pigs were observed which developed no febrile reaction. Convalescent specimens of serum from both of these guinea pigs showed the presence of epidemic complement fixing antibodies and the animals proved immune on challenge. The other 48 animals in this series displayed fever after primary inoculation, developed complement fixing antibodies, and were subsequently immune when challenged. Further evidence was acquired on the development of complement fixing antibodies in guinea pigs with an inapparent infection when 30 animals were inoculated with small doses of epidemic typhus rickettsiae. Seventeen of the animals responded in the usual manner. Thirteen individuals in the group developed no febrile reaction after inoculation but epidemic complement fixing antibodies were demonstrated in samples of serum obtained one month later from each of the thirteen. Finally, each of these animals proved to be immune when challenged with epidemic typhus.

Rocky Mountain Spotted Fever. Thirteen guinea pigs were bled for serological studies and then inoculated intraperitoneally with 1 cc amounts of a pool of infectious blood obtained from guinea pigs acutely ill with a mild strain of Rocky Mountain spotted fever. A febrile period of from 2 to 9 days followed an incubation period varying from 2 to 6 days. The guinea pigs were bled 14 days after the temperature returned to normal. Serum from each animal contained complement fixing antibodies that reacted with the Rocky Mountain spotted fever antigen and none that reacted with the epidemic or murine antigen. All preinoculation specimens were negative with the 3 antigens. Data from this experiment are summarized in Table III.

Discussion. The identification of strains of epidemic and murine typhus or Rocky Mountain spotted fever based on animal reactions alone presents certain experimental difficulties. This has been illustrated when guinea pigs were inoculated at the same time with the same amount of a pool of infectious material. Irrespective as to whether the guinea pig develops evidence of disease as expressed by a febrile reaction or scrotal swelling, or an inapparent disease without these reactions, specific complement fixing antibodies develop in early convalescence. The use of the complement fixation reaction, likewise, permits the detection of those animals that represent missed infections or those that develop fever from non-specific causes. The use of the complement fixation method for strain identification is specific, rapid and inexpensive.

Summary. 1. Guinea pigs inoculated intraperitoneally at the same time with the same amount of a pooled infectious suspension of tunica vaginalis washings, Wilmington strain of murine typhus, developed three types of reaction following a variable period of incubation: (a) fever of variable duration and scrotal swelling, followed by the appearance of specific murine complement fixing antibodies; (b) fever of variable duration and no scrotal swelling followed by the appearance of specific murine complement fixing antibodies; and (c) no febrile reaction or scrotal swelling but followed by the appearance of specific murine complement fixing antibodies (inapparent infection). All guinea pigs showing one of these three types of response were proven immune when challenged subsequently.

2. Guinea pigs inoculated intraperitoneally at the same time with the same amount of pooled infectious brain suspension, Breinl strain of epidemic typhus, developed 3 types of reaction following a variable period of incubation: (a) febrile period of variable duration followed by the appearance of specific epidemic complement fixing antibodies; (b) no febrile reaction but followed by the appearance of specific epidemic complement fixing antibodies (inapparent infection); and (c) no febrile reaction followed by no complement fixing antibodies. This latter group are regarded as "misses" for these guinea pigs were not immune when challenged while those of group a and b were immune.

3. Specific murine or epidemic complement fixing antibodies were demonstrated after the inapparent infections with these 2 types of typhus.

4. Guinea pigs inoculated at the same time with the same amount of pooled infectious blood of Rocky Mountain spotted fever (mild strain) exhibit a febrile reaction of variable duration following a variable period of incubation. In all instances specific complement fixing antibodies to Rocky Mountain spotted fever were demonstrated in convalescent specimens of serum and no complement fixing antibodies with a murine or epidemic antigen.

Conclusion. The specific complement fixation test, using purified rickettsial suspensions as antigen, is recommended as a routine test to differentiate infections in guinea pigs caused by rickettsiae of murine and epidemic typhus and Rocky Mountain spotted fever.

15232

Studies on Chancroid. IV. The Ducrey Bacillus: Growth Requirements and Inhibition by Antibiotic Agents.*

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The classification of the Ducrey bacillus in the genus *Hemophilus* appears to be based largely on its morphology and on the fact that it grows best in a medium containing blood. Actually the growth requirements and biology of this organism have received very little study. The present article is a report of some observations made in the course of a clinical and laboratory investigation of chancroid. The data presented support the view that there are important differences between the Ducrey bacillus and other members of the genus *Hemophilus*.

Materials and Methods. The base medium used was proteose peptone broth (Difco). Blood was obtained from rabbits by cardiac puncture. Defibrination was accomplished by shaking with glass beads. Erythrocyte suspensions were prepared by washing repeatedly with large volumes of normal saline, until a negative Pandy test was obtained on the supernatant fluid.

X factor was prepared as follows: The clot

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TABLE I.
Growth of Ducrey Bacillus, *H. influenzae*, and *H. parainfluenzae*, in Media Containing Whole Blood and X and V Factors.

Organism	Base medium, plus			
	X factor	V factor	X and V factors	Whole blood
Ducrey No. 1	0	0	0	++++
No. 2	0	0	0	++++
No. 3	0	0	0	++++
No. 4	0	0	0	++++
No. 5	0	0	0	++++
No. 6	0	0	0	++++
H. influenzae 6208	0	0	++++	++++
” ” I	0	0	++++	++++
” ” II	0	0	++++	++++
H. parainfluenzae 7901	0	++++	++++	++++

Base medium: Proteose peptone broth (Difco).
Whole blood: Defibrinated rabbit blood (concentration 10% in base medium).
H. influenzae 6208, *H. parainfluenzae* 7901: American Type Culture Collection.
H. influenzae I, *H. influenzae* II: Type a strains recently isolated from patients with influenzal meningitis.
This table represents findings after more than three successive subcultures.
Readings based on microscopic examination of stained smears of cultures, after 48 hours' incubation at 34°C.

from 40 ml of rabbit blood was infused in 8 ml of normal saline at room temperature for one hour. This was then boiled for 5 minutes, and filtered through paper. The filtrate was autoclaved for 15 minutes at 15 pounds pressure. This material was added to the base medium in a concentration of 4% to provide an adequate supply of X factor.

V factor was prepared as follows: 20 g of baker's yeast were emulsified in 80 ml of distilled water, by rubbing in a glass mortar. The reaction was adjusted to pH 4.6 by addition of N/1 HCl. The mixture was boiled for 10 minutes and then centrifuged at 2000 r.p.m. for 20 minutes. The clear supernatant material was used as a source of V factor, being added to the base medium in a concentration of 10%.

All cultures were incubated at 34°C. Liquid media were distributed in 1-ml quantities, and cultures were inoculated with 1 loopful of material.

Requirement of X and V Factors. Hemophilus influenzæ has been shown to require for growth 2 agents which are present in erythrocytes. One of these, originally called the X factor, is heat-stable and is known to be hemin. The other, called V factor, is heat-labile, and is replaceable by coenzyme I or II.[1] *H. parainfluenzæ* requires only the V factor.

Six strains of Ducrey bacillus were tested for growth in the presence of X and V factors. Three strains of *H. influenzæ* and one of *H. parainfluenzæ* were included in the experiment. The results of the test are shown in Table I. It will be seen that all 10 organisms were able to grow in the medium in the presence of defibrinated blood. On the other hand, addition of X factor alone was not sufficient to support growth of any of the 10 organisms. *H. parainfluenzæ* was able to grow in a medium containing V factor alone. The 3 strains of *H. influenzæ* and *H. parainfluenzæ* all grew in a medium containing both X and V factors, but none of the Ducrey strains would grow in it. Thus, although the Ducrey bacillus resembles true members of the genus *Hemophilus* in that growth is supported by the presence of blood in the medium, it differs by inability to grow in a medium containing only X and V factors. One must conclude from this either that the X and V factors are not the substances essential for growth of the Ducrey bacillus, or that some other factor which is present in whole blood in addition to X and V factors is needed for growth.

Growth in Media Containing Cells Alone, or Serum Alone. The constituents of whole blood necessary for the growth of *H. influenzæ* and *H. parainfluenzæ* are present in the ery-

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[1] Lwoff, A., and Lwoff, M., *Proc. Roy. Soc., London, Series B*, 1937, **122**, 352, 360.

TABLE II.
Growth of Ducrey Bacillus, *H. influenzae* and *H. parainfluenzae* in Media Containing Blood Serum or Washed Erythrocytes.

Organism	Base medium, plus	
	Blood serum	Washed erythrocytes
Ducrey No. 1	++++	++++
No. 2	++++	++++
No. 3	++++	++++
No. 4	++++	++++
No. 5	++++	++++
No. 6	++++	++++
H. influenzae 6208	0	++++
” ” I	0	++++
” ” II	0	++++
H. parainfluenzae 7901	0	++++

This table represents findings after more than 3 successive subcultures.

Readings based on microscopic examination of stained smears of cultures, after 48 hours' incubation at 34°C.

throcytes, not in the blood serum. It is known, however, that the Ducrey bacillus will grow in a medium enriched only with blood serum. A demonstration of this is the experiment shown in Table II. The concentration of serum required for growth of Ducrey bacilli appears to be relatively high. Optimum growth was obtained in media containing from 25 to 50% serum although some of the strains tested survived when the concentration was reduced to as low as 10%. The fact that Ducrey bacilli will grow in serum broth, a medium which will not support growth of either *H. influenzæ* or *H. parainfluenzæ*, together with the evidence in the preceding section, suggests that neither X nor V factor is essential for growth of the Ducrey organism.

The Ducrey bacillus can also be cultivated in a base medium enriched only with erythrocytes. The volume of erythrocytes necessary is relatively large: 10% or greater.

It would appear that enrichment with whole blood provides a better medium for growth of the Ducrey bacillus than is obtained with either cells or serum alone since growth can be maintained indefinitely in a base medium containing only 3% of defibrinated blood. It is probable, therefore, that whole blood contains more than one growth substance utilized by Ducrey organisms, and that these substances are present in both erythrocytes and serum. Further evidence bearing on this will be given in the next section.

Heat Stability of Growth Factors in Erythrocytes and Serum. The substances in rabbit blood serum essential for growth of Ducrey bacilli are not destroyed by heating at 100°C for 30 minutes. Twenty per cent serum broth heated in this manner will support the growth of Ducrey bacilli through repeated transfers without apparent change. Growth does not occur, however, in serum broth which has been autoclaved at 15 pounds pressure for 20 minutes.

The growth-supporting properties of erythrocytes are more easily destroyed by heat than are those of serum. Ten per cent erythrocyte-broth would not support growth of any of 6 strains of Ducrey bacillus after heating at 65°C for 30 minutes. Four of the 6 strains tested would not grow in this medium after it had been heated at 60° for 30 minutes.

The difference in heat stability of the growth factors in erythrocytes and serum provides further evidence that the agents present in the two constituents of blood are not the same.

Importance of Moisture for Growth of Ducrey Organisms on Solid Media. A striking peculiarity of members of the Ducrey group is their dependence on moisture for growth on the surface of a solid medium. We have discussed this elsewhere, in an article dealing with the cultural diagnosis of chancroid.[2] Usually no visible growth occurs on the surface of an inoculated blood agar plate placed in an ordinary incubator. On the other hand, a plate which has received a similar inoculum and which is incubated at the same temperature inside a closed jar containing a moist sponge will show a good growth of Ducrey colonies at the end of 24 hours. *H. influenzæ* and *H. parainfluenzæ* show no such rigid dependence on moisture in the air.

Satellite Phenomenon. Most members of the genus *Hemophilus* exhibit more luxuriant growth on the surface of a solid medium in the vicinity of colonies of certain other bacteria, particularly staphylococci. This enhanced growth is called the "satellite phe-

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[2] Beeson, P. B., and Heyman, A., *Am. J. Syph., Gon. and Ven. Dis.*, in press.

TABLE III.
Growth of Ducrey Bacillus, *H. influenzae,* and *H. parainfluenzae* in Media Containing Varying Concentrations of Tyrothricin.

	Dilution of tyrothricin						
Organism	4000	8000	16000	32000	64000	128000	0
Ducrey No. 1	0	0	0	+	+++	+++	++++
No. 2	0	0	0	0	++	++++	++++
No. 3	0	0	0	0	++	+++	++++
No. 4	0	0	0	0	0	++	++++
No. 5	0	0	0	++	+++	+++	++++
No. 6	0	0	0	++	++	++++	++++
H. influenzae 6208	0	++	++++	++++	++++	++++	++++
,, ,, I	0	++	++++	++++	++++	++++	++++
,, ,, II	0	+++	++++	++++	++++	++++	++++
H. parainfluenzae 7901	+	++	++++	++++	++++	++++	++++

Medium: 10% defibrinated blood in base medium.

This table represents findings after more than three successive subcultures.

Readings based on microscopic examination of stained smears of cultures, after 48 hours' incubation at 34°C.

nomenon." It is thought to be due to liberation of growth substances by the staphylococci. No such change is noted in Ducrey colonies, whose gross appearance is not altered by proximity to staphylococcus colonies on the surface of a solid medium.

Dialysis of Growth Factors. Repeated attempts were made to demonstrate the growth factors for Ducrey organisms in dialysates of either defibrinated rabbit blood or fresh rabbit serum. Blood and serum were dialyzed against distilled water, physiologic salt solution, and proteose peptone broth. Sterilization of dialysates was accomplished by Seitz filtration, a procedure which does not affect the growth-stimulating properties of serum broth. None of the dialysates obtained would support growth, either alone or when added to the base medium.

Bacteriostasis by Antibiotic Substances. The 3 strains of *H. influenzæ* and 1 strain of *H. parainfluenzæ* were compared with 6 strains of Ducrey bacillus in respect to inhibition of growth by penicillin, tyrothricin, streptomycin,[†] and streptothricin. Other workers have already reported that penicillin exerts a pronounced bacteriostatic effect on Ducrey organisms.[3] We have repeated that work and our findings agree entirely. In addition we find a similar difference in respect to inhibition by tyrothricin. The results of a test are shown in Table III, where it will be observed that the typical *Hemophilus* organisms were able to grow in much greater concentrations of tyrothricin than was the case with strains of Ducrey bacillus. This is further evidence of a basic difference between Ducrey bacilli and true *Hemophilus* organisms. In respect to streptothricin and streptomycin, no significant difference in susceptibility was found.

Summary. In a comparative study of several strains of Ducrey bacillus with *H. influenzæ* and *H. parainfluenzæ* a number of significant biologic differences have been noted. The Ducrey bacillus will not grow in a medium which supplies both the X and V factors. Unlike these other members of the genus *Hemophilus,* the Ducrey bacillus will grow in a medium enriched only with blood serum. It will also grow in a medium enriched only with erythrocytes. The agents in serum and in erythrocytes which support growth of the Ducrey bacillus differ in heat stability; that in serum withstands 100°C but not autoclaving, while that in erythrocytes is inactivated by heating to 65°C. The addition of both erythrocytes and serum to the base medium provides a more favorable medium for the growth of the Ducrey bacillus than either one alone. Ducrey colonies will not develop on the surface of a solid medium unless the humidity of the atmosphere is very high. The "satellite phenomenon" is not observed in colonies of Ducrey bacillus. Attempts to separate Ducrey growth factors from whole blood or serum by dialysis have not been successful. The Ducrey bacillus differs significantly from *H. influenzæ* and *H. parainfluenzæ* in susceptibility to inhibition by penicillin and by tyrothricin.

[†] Courtesy of Dr. S. A. Waksman.

[3] Mortara, M., Feiner, R. R., and Levenkron, E., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 163.

Miss Elizabeth Roberts gave technical assistance in this work.

15233

Muscles Consisting of a Single Motor Unit After Poliomyelitis.

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During an investigation concerning the possible increase of muscle force in paretic muscles by means of increased motor nerve branching[1] the action potentials of more than 1000 different paretic muscles were obtained. Among these the records of 36 muscles in 26 different patients were remarkable for their simplicity and they resembled closely records produced by single motor units in fully[2,3,4] or partially innervated muscles,[5] though no measures were taken to insure the recording of units. The behavior of these muscles was analyzed by repeated tests.

Methods. In routine electrograms, the patient contracted the muscle as strongly as possible for 2 or 3 seconds and then released the tension quickly. Electrodes, consisting of solder pellets of 5 mm diameter were attached to the skin with tape, electrode paste being used to insure a good contact. The action potentials were amplified and recorded by a Matthews oscillograph. Nearly all of the muscles to be described were found in patients who had undergone the treatments intended to enhance the nerve branching.[1] In all cases the paresis was at least of one year standing.

Results. The short lasting, strong contractions of all muscles under consideration were accompanied by a single row of action potential spikes, (Fig. 1) which were in general, rather evenly spaced and often grew considerably in height, especially the first 10 spikes. In by far the majority of the cases the voluntary control of the muscle was good, in only a few were there signs of poor control as evidenced by an irregular and low frequency of the spikes and difficulty in maintaining the contraction even for a short time.

The maximum frequency of the spikes reached in this way has been measured for all these muscles and was found to vary between 20 and 60 per second, the mean being 40. In accordance with the data of Gibson and Mills[3] it was observed that 2 or even 3 spikes could follow each other closely, (0.01 second), after which there was usually a somewhat longer delay before the appearance of the next spike. Therefore the maximum frequency was computed by using the time interval of at least 10 consecutive spikes. Since most of the 15 different muscles used in the investigation are represented only a few times and these are only larger muscles in which the influence of the contraction of neighboring muscles on the electrogram is negligible, the material is not very suitable to show whether or not different muscles have different inherent maximum frequencies.[5] It may be significant, however, that the deltoid which is represented 5 times, showed a maximum of 60 in 3 and of 40 in 2 of these cases, and has thus a mean frequency greater than that of the mean maximum given above.

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[1] Billig, H. E., van Harreveld, A., and Wiersma, C. A. G., *J. Neuropath. and Exp. Neurol.*, 1946, in press.

[2] Smith, O., *Am. J. Physiol.*, 1934, **108**, 629.

[3] Gilson, A. S., and Mills, W. B., *Am. J. Physiol.*, 1941, **133**, 658.

[4] Denslow, J. S., and Hassett, C. C., *Am. J. Physiol.*, 1943, **139**, 652.

[5] Lindsley, D. B., *Am. J. Physiol.*, 1935, **114**, 90.

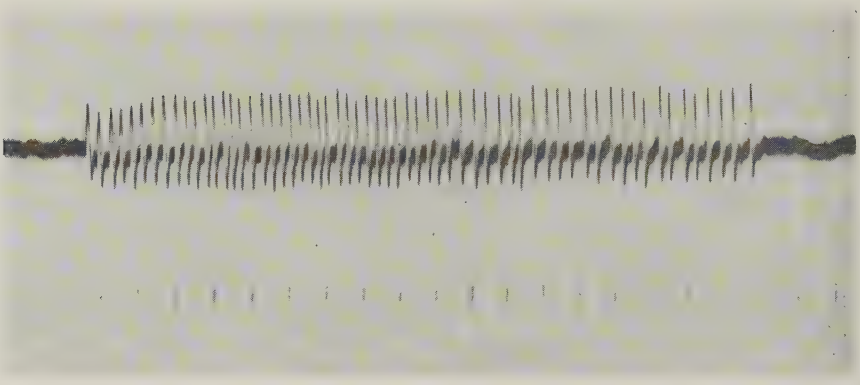

Fig. 1.

Test contraction of a rectus femoris; untreated patient. Facilitation 25%, diphasic, frequency 40 per second. As in all following pictures skin leads were used. Time 1/10 sec.

The growth of the spikes of the action potentials during the contraction was present in all cases and often very pronounced. In records in which the action potentials were practically monophasic this increase was usually larger than with di- or polyphasic spikes. In the latter there was an increase of more than 50% in 14 of the 27 records, and in only 1 record was the increase as small as 20%. In the monophasic group 13 out of 26 records showed an increase of more than 100% and all but one of more than 50%. One muscle was outstanding in the amount of facilitation obtained. The first top was always very small and the later ones were at least 10 times as large. (Fig. 2).

The amount of facilitation was found to be dependent on several factors, in any one muscle. Relatively little difference was encountered when on different days similar contractions were performed with similar electrode placements, unless the state of fatigue of the muscle was different. At any given time with the electrodes fixed, the most important factor was the frequency of the impulses. Up to a certain limit the growth is faster and larger the more rapidly the spikes follow each other. However, above a certain frequency, which seems to differ for different muscles, the potentials become smaller. This does not interfere with the facilitation process, any spike which happens to be separated by a sufficient time interval from a foregoing one will be large. In a fatigued muscle the growth is much smaller and may, soon after the start of the contraction, stop and be replaced by a decrease in height, even when the frequency is low. In a few instances the muscle was in tone both before and after the test contraction. In these cases little or no facilitation took place, which is explained by the supposition that during the tonic period facilitation had developed to such an extent, that the increase in frequency has no longer a pronounced effect.

In a limited number of experiments the contractions were maintained for long times. It was found that in most cases the spikes after their initial facilitation start to decline confirming Smith[2] and may become very small indeed. (Fig. 2). In others the frequency becomes very irregular which in turn gives irregular action potential heights. In one case 5 minutes contraction did not result in any noticeable decrease. Though the rate of the spikes was rather low, (± 25), other muscles with even lower rates showed profound fatigue after much shorter times.

Once a muscle has been fatigued, the result remains noticeable for a long time. During the first minutes after relaxation the action potentials remain small, after longer rest periods the growth is at first normal but diminishes soon and the maximum height is never reached. This may well play a part in the difference of facilitation obtained in different muscles. In one instance a muscle

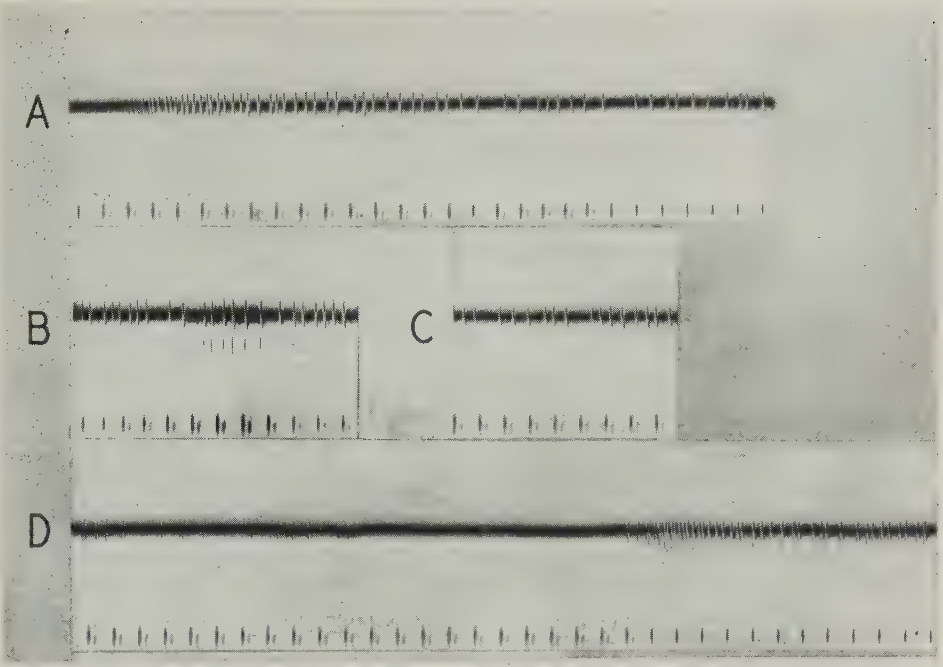

FIG. 2.

Deltoid, treated. Long lasting contraction. A. Start. Notice pronounced facilitation and triphasic spikes. B. After 15 seconds, maximum facilitation is reached. C. After 30 seconds, the height of the spikes is diminished. D. After 90 seconds. The contraction ends with muscle action potentials just visible. After 1 second rest they reappear and facilitate, but diminish quickly. Time 1/10 second.

which had been rested for a long time reached maximum peak height after one minute, whereas the same muscle normally reached its maximum after 30 seconds and already began to diminish in height at one minute. If, therefore a patient has used the muscle to a great extent, *e.g.* in walking, the rest provided during the investigation time may not be long enough to overcome the after-effects of this use.

In another series of experiments repeated contractions of very short duration were studied. In such contractions each ensuing contraction starts with a higher action potential than the previous one (Fig. 3) which shows that purely mechanical factors are not responsible for the increase in height. In this type of contraction the frequencies reached tend to be higher than during the "standard" contractions and may become high enough to seriously interfere with the gradual growth of the action potentials. (Fig. 3). At least in these muscles it seems possible to obtain frequencies in the nerve fibers which are too high for the muscle fibers to follow normally.

In a number of selected cases mechanograms were obtained from these muscles by the application of a heart apex beat recorder over the contracting belly. In some cases but not in all, single twitches caused by a single impulse were obtainable,[3] in these it was found that higher contractions and action potentials occurred after previous facilitation by a tetanic contraction. Summations of 2 and more impulses could be nicely demonstrated with this method. (Fig. 4).

In some of the cases needle electrodes were put into the substance of the muscle. The resulting action potentials differed only in voltage from those obtained from the intact skin.

Discussion. As in all cases of human muscles it is not certain that the data presented are caused by the action of single motor units.[3] Especially the growth of the

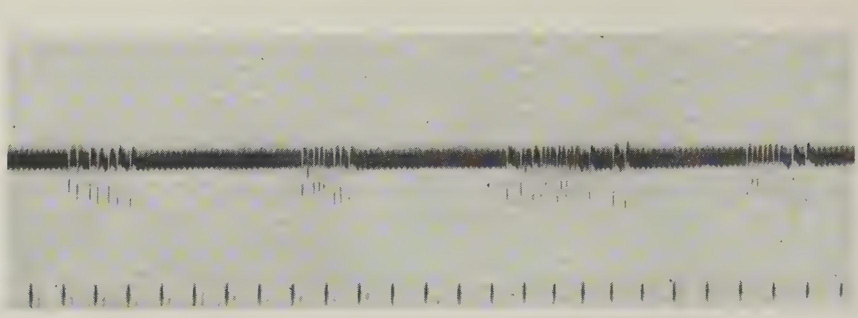

FIG. 3.

Lower peroneal, treated. Series of short lasting contractions. In the first of the series the facilitation is regular; in the following three the frequency is higher and the spikes are irregular. Each of the later contractions starts with a higher spike than the previous one. Time 1/10 sec.

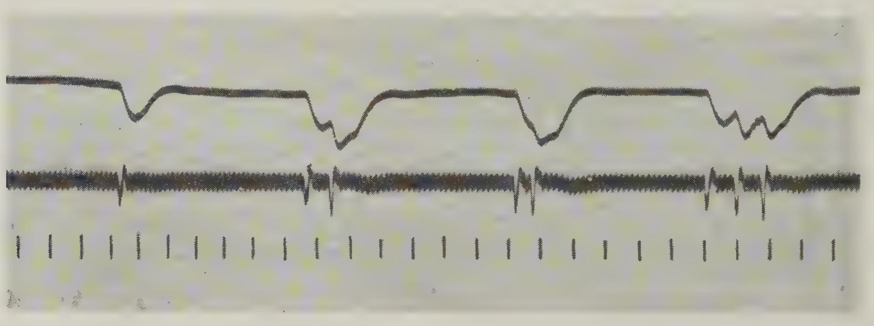

FIG. 4.

Gluteus maximus, treated. A series of voluntary twitches, the first of which is single. Upper line, mechanogram, middle electrogram, lower time 1/10 sec.

action potentials which from the evidence presented cannot be ascribed to artifacts, might well be considered as against the presence of single motor units. The alternative would be that a number of nerve fibers would always fire at the same time, but would have different thresholds. Such a mechanism might possibly be brought about by the connection of a number of motorneurons with a single pyramidal fiber. If this were the case the number of motorneurons so connected should be large in order to explain the smooth increase of the growth of the potentials. No evidence was ever obtained which showed that these hypothetical central units could split themselves into component parts, and they would therefore form a serious handicap to the amount of central control of the contractions. Furthermore in all cases the contracting part of the muscle was a single band, whereas if different axons were involved one would expect that several contractile bands would occur in at least some of them.

If then these bands are considered as single units it remains to be discussed what the growth in the action potentials represents. There are two likely explanations. The first is that facilitation represents the increased spreading of the action potential over the muscle fibers. In freshly reinnervated muscles such a process might well take place, the first impulse of a series would be limited to the neighborhood of the end plate and successive impulses would spread more and more to the ends of the muscle fibers. However, as recovery went on, it would be expected that facilitation become less and less pronounced. This was not observed; in patients which have been studied for one year or more no difference in the amount of facilitation was found between the first and the last test. The other, and perhaps more likely possibility is that only part of the muscle fibers respond on a single nerve impulse and that considerable facilitation is necessary before all fibers contract with every impulse. Such a mechanism has been claimed to be normal in avian muscles.[6] Some experiments were performed with this possibility in mind. The patients were given prostigmine, intramuscular in the acute and orally in other experiments. The dosage was sufficient to cause distinct signs of the action of the drug, of which especially the increase in the size of the action potentials in the acute cases should be mentioned. However no noticeable effect on the degree of facilitation was observable. It must therefore remain at this time undecided by which mechanism the facilitation is brought about.

Summary. It was found that in some patients with chronic poliomyelitis very weak muscles can be found which seem to consist of one remaining motor unit. A study of their properties has been made by recording the action potentials through skin leads.

The author is indebted to Dr. A. van Harreveld and Dr. H. E. Billig for their help and for their interest in this research.

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[6] Brown, G. L., and Harvey, A. M., *J. Physiol.*, 1938, **93**, 285.

15234 P

Development of Brucella Agglutinins in Humans Following Vaccination for Cholera.*

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During the course of a survey for brucella agglutinins made on sera discarded from the Wassermann laboratory (C. W. E., N. B. McC. and G. A. B.), a blood bank donor was found to have a brucella agglutinin titer of 1:200. This individual was a healthy male who, 3 months previously, had been experimentally vaccinated by one of us (W. B.) with U. S. Army cholera vaccine. The possibility that this finding might be more than a coincidental occurrence led us to examine the stored sera from the cholera studies for brucella agglutinins.

Methods and Material. Thirty-four individuals were given either regular U. S. Army cholera vaccination or varying amounts of an experimental cholera vaccine consisting of extracted cell residue. Whereas the Army vaccine contained both H and O antigens, the experimental vaccine contained primarily the O antigen. The vaccines were given in 2 doses of 0.5 and 1.0 cc with an interval of one week between inoculations. Sera were collected before immunization and at 1, 2, 5 and 9 weeks after completion of the vaccination. Brucella agglutinin titers were determined by the rapid slide method of Huddleson, using the standard dilutions from 1:25 to 1:500. The subjects were divided into 4 groups:

Group I—7 subjects, routine U. S. Army cholera vaccination, 8,000 million organisms per cc.

Group II—8 subjects, experimental vaccine, 8,000 million organisms per cc.

Group III—10 subjects, experimental vaccine, 24,000 million organisms per cc.

Group IV—9 subjects, experimental vaccine, 40,000 million organisms per cc.

Five post-immunization sera from 3 individuals were further studied by reciprocal agglutinin absorption tests, using as antigens a live virulent strain of *Br. abortus* and a live 24-hour culture of *Vibrio comma.* After absorption with brucella, the sera were tested

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* Aided in part by a grant from Swift and Company. A part of the work in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

TABLE I.
Brucella Agglutinins in Sera of Subjects Immunized with Regular U. S. Army Cholera Vaccine.

Subject No.	Pre-immunization sera	Time after last inoculation 1	2	5	9 weeks
7	—	1:500P	1:200	1:200P	1:200P
10	—	1:500P	1:500	1:200	1:200
16	1:25	1:25	1:50	1:50	*
21	—	—	—	—	—
30	—	—	1:100P	—	—
32	—	1:200P	1:100P	1:50	1:25
38	—	1:100P	1:25	—	—

— Negative.
P Partial agglutination (usually complete agglutination in the next lower titer).
* Serum not available.

with cholera antigens[1] containing HO and containing only O antigen.

Results. The brucella agglutinin titers of the 7 individuals in Group I who received the U. S. Army type of cholera vaccination are presented in Table I. One of the subjects had a pre-immunization titer of 1:25, all the others being negative. Following immunization, all except one developed brucella agglutinins in titers ranging from 1:50 to 1:500. In 3 subjects, brucella agglutinins were still present at the time of the last collection of sera (9 weeks).

In Groups II, III and IV receiving varying amounts of the experimental cholera vaccine, none of the 27 individuals showed any pre-immunization brucella titers, and in only 2 subjects, low-grade post-immunization brucella agglutinin titers developed. These were both in Group IV receiving the largest amount of the vaccine, and the titers of 1:25 and 1:50 P were very transient, disappearing by the 9th week.

In the reciprocal agglutinin absorption tests, absorption with living 24-hour culture of *Vibrio comma* removed all brucella agglutinins. Sera absorbed with a live, virulent strain of *Brucella abortus* subsequently agglutinated formalinized *Vibrio comma* (HO) in markedly lowered titers.[†] (Serum E7, before absorption 1:1000, after absorption 1:100; Serum B32, before 1:5000, after 1:200; Serum C10, before 1:5000, after 1:200; Serum C32, before 1:5000, after 1:500; Serum D7, before 1:2000, after 1:2000.[†]) Agglutination tests with a boiled suspension of *Vibrio comma* (O antigen) were not significantly different from the pre-absorption tests.

All 34 of the vaccinated subjects developed cholera agglutinins in high titers which persisted during the 9-week post-immunization observation period. The magnitude of the cholera agglutinin response was approximately the same following the two types of vaccine. In the 3 groups receiving the varying amounts of the experimental vaccine, augmented cholera agglutinin titers did not accompany increasing amounts of vaccine. In fact, Group IV receiving the largest amount of vaccine had a somewhat poorer average cholera agglutinin response than Groups II and III receiving smaller amounts. These data will be reported in detail elsewhere.[1]

Discussion. Wong and Chow[2] reported that in 8 rabbits injected intravenously or subcutaneously with heat-killed suspensions of *Vibrio comma*, brucella agglutinins developed in unspecified titers. Of 6 humans inoculated subcutaneously or intravenously with a typhoid-cholera vaccine, 4 developed agglutinins against *Brucella abortus* in titers of 1:40 or less. Presumably, the vaccines used had lost at least some of the H antigen during heating.

The agglutination test for brucella is the most commonly used diagnostic procedure for brucellosis. Although it is well known that proven cases of the disease may at times present little or no agglutinin response, the diagnosis of brucellosis is frequently made on the basis of low-grade titers without adequate supporting data. Many clinicians consider brucella agglutinin titers in dilutions of 1:500 as almost certainly diagnostic of active infection. The development of titers as high as this following vaccination for cholera may well be expected to introduce further confusion in the clinical interpretation of this test. Cholera vaccination has been infrequently used in this country prior to the war; but large numbers of veterans are returning from the Pacific and Asiatic theaters who have been subjected to this immunization.

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[1] Burrows, William, unpublished data.

[†] In one serum, D7, the post-absorption titer was not lowered. Insufficient serum remained for further tests.

[2] Wong, D. H., and Chow, C. H., *Chinese Med. J.*, 1937, **52**, 591.

Studies are underway to determine the duration of the persistence of brucella antibodies following vaccination for cholera, and the possible reappearance of antibodies in the circulating blood as an anamnestic phenomenon. The possible effect of other types of immunizations on brucella antibody stimulation must also be considered.

Our preliminary data presented here indicate that there is an H antigen in *Vibrio comma* which is present also in brucella. Studies are in progress to elucidate further the antigenic interrelationships of these organisms.

15235 P

Excitability of Muscle to Direct and Indirect Stimulation During Prolonged Direct Stimulation.*

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Although electrical stimulation is used widely in diagnosis and in physical therapy and is the only practical procedure to test muscle function in animals, the effects of prolonged direct stimulation have not been studied extensively.

Alternating 60-cycle current was used in 3 independent but *in phase* circuits for stimulation through 3 pairs of electrodes, the first pair being inserted into the knee and tendon of the gastrocnemius muscle *in situ* of anesthetized rats (nembutal) and cats (dial), (D_1 electrodes); the second pair (D_2) inserted into the muscle itself at its upper and lower ends; the third pair (I) being employed for stimulation of the peripheral end of the cut sciatic nerve. The strength of the stimulus was adjusted with a rheostat and measured by a volt meter; in addition, the resistance in the muscle was measured by means of an oscilloscope in a shunt parallel to the muscle. Stimulation was continued either with D_1 or with D_2 until the muscle length reached the prestimulus resting value. During and after the continued stimulation, brief tetanic stimuli were applied at regular intervals through the 2 other pairs of electrodes. In all cases maximum stimuli were applied through a 10,000-ohm resistance placed in series with muscle and nerve. The muscle was loaded with 200 g and contraction was isotonic. Before continued stimulation the effect of summation was tested for all combinations of D_1, D_2, and I. There was no summation between D_1 and I, or D_2 and I, and a tendency to slight summation of $D_1 + D_2$.

Steel, platinum, silver, and nonpolarizable ($Cu\text{-}CuSO_{4_{1.0M}}$ - $K_2SO_{4_{sat.}}$ - $NaCl_{sat.}$ $NaCl_{0.9\%}$-Tissue) electrodes were employed in various experiments. There was no essential difference in the results to be reported. Forty-seven experiments were performed in rats and 3 experiments in cats.

With continued stimulation through D_1, there is no response to D_2 or I at the very beginning, but after a few minutes a marked

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

response to D_2 appears, while response to I is absent. The absence of response to I cannot be due to the absence of muscle fibers available for stimulation, since response to D_2 is present. After 10 to 20 minutes of stimulation with D_1, response to I begins to reappear and to increase gradually in size, but usually remains somewhat smaller than the D_2 response. After "fatigue" (*i.e.*, approach to the original resting length) the responses to both I and D_2 are very marked and may approach the height of initial contraction. If at this stage continued stimulation through D_2 or I is superimposed on the uninterrupted D_1 stimulation, a large tetanic contraction occurs, which however returns to the baseline much faster than it would have had the muscle been stimulated solely through these electrodes. If the stimulation of D_2 or I is interrupted at this moment, while D_1 stimulation continues, the response to D_2 or I stimulation recovers.

When continued stimulation is applied through D_2, the responses both to D_1 and I stimulation appear within a short time and are present throughout the development of fatigue.

The continued stimulation through D_1 is accompanied by an increase in muscle resistance, as indicated by the oscilloscope studies. This increased resistance is related in time of appearance to the return of response to indirect stimulation. The effect of D_2 stimulation was less marked. Investigations by means of crossed D_1 and D_2 electrodes revealed that the difference between continued D_1 and D_2 stimulation is due to the lower D_1 electrode inserted into the tendon.

The results described above could be reproduced in all experiments with only one exception. Experiments on cats were confirmatory. It appears doubtful whether the diminishing response to continued direct stimulation may be regarded as a simple fatigue phenomenon. Rather it appears more likely to be due to some kind of rectification or polarization in the tissues, however, not to polarization of the electrodes themselves.

The more intimate mechanism of these effects is being investigated. The effects themselves appear to have some practical significance in connection with the therapeutic use of alternating currents in muscle stimulation. They may also be of importance in relation to fundamental aspects of excitability and fatigue in striated muscle.

15236

"Folic Acid" Active Compounds in the Nutrition of the Monkey.*

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Two years ago we reported[1] that the norite eluate concentrate of Hutchings *et al.*,[2] a rich source of "folic acid," was active in producing normal growth and preventing leucopenia in monkeys given a synthetic ration containing all the B vitamins available in pure form. Hutchings *et al.*[3] had shown previously that this concentrate contained factors necessary for the normal development of chicks. More recently Briggs *et al.*[4] concluded that the norite eluate contains at least 3 factors: folic acid necessary for the growth of *Lactobacillus casei;* vitamin B_{10}, necessary for normal feathering; and vitamin B_{11}, necessary for normal growth in chicks. They[5] further demonstrated that fractions high in vitamin B_{10} and

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We wish to acknowledge our indebtedness to Merck and Co., Rahway, N.J., for the crystalline vitamins; to Wilson Laboratories, Chicago, Ill., for the various liver preparations; to Dr. J. J. Pfiffner of Parke, Davis and Co., Detroit, Mich., for the crystalline B_c and B_c conjugate; to Dr. B. L.

B_{11} activity but low in folic acid can promote excellent growth and feathering in the chick. Pfiffner *et al.*[6] isolated a crystalline compound from liver which they called vitamin B_c and found that it has both growth-promoting and hemopoietic properties for the chick. Since vitamin B_c also has growth-promoting properties for *Lactobacillus casei,* these workers suggested that vitamin B_c, Hutchings' norite eluate factor, and Williams' folic acid[7] are probably identical. Binkley and coworkers[8] isolated from yeast a B_c conjugate which has vitamin B_c activity for the chick but no activity for the bacterium. However, upon enzymatic digestion the B_c conjugate becomes active as a source of B_c for the *Lactobacillus casei.* Day and coworkers[9] reported that highly purified *Lactobacillus casei* factor was effective in treating vitamin M-deficient monkeys. In this paper we wish to report the relative effectiveness of vitamin B_{10} and B_{11} concentrates, vitamin B_c, vitamin B_c conjugate and the synthetic *Lactobacillus casei* factor when used in place of the norite eluate concentrate in our purified rations for the monkey.

Experimental. The method of handling the monkeys has been described previously.[10] The M-2 basal diet consisting of sucrose 73, Labco casein 18, salts IV 4, corn oil 2, cod liver oil 3 was fed *ad libitum* and supplemented with adequate amounts of ascorbic acid, thiamine, riboflavin, nicotinic acid, pyridoxine, choline, pantothenic acid, inositol, *p*-aminobenzoic acid, and biotin.

A liver fraction high in B_{10} and B_{11} activity but low in "folic acid" activity was prepared according to the directions of Briggs *et al.*[5] In brief this method consists of dialysing a concentrated superfiltrol eluate[2] in a cellophane bag against cold water for 96 hours. The residue is saved and placed in a Visking cellulose casing and dialysed against hot distilled water. The dialysate is saved and concentrated so that 1 cc of the final solution is equivalent to 10 g of the original solubilized liver extract (fraction L).

The dialysate was assayed for B_{10} and B_{11} activity using chicks and for "folic acid" using the microbiological method. Using as a standard (100%) the growth and feathering of chicks fed a ration containing 2% solubilized liver (fraction L), one cc of the dialysate showed 95% B_{10} and B_{11} activity. Assay with *Streptococcus lactis* showed that 1 cc of the dialysate contained less than 10 γ folic acid using crystalline vitamin B_c as the standard.

Studies with B_{10} *and* B_{11} *Concentrates.* Three monkeys, No. 217, 218, and 219, were placed on the basal ration and given daily 1 cc of the concentrated dialysate. The monkeys grew at a slow rate for 8 weeks after which they began to lose weight. The supplement was then increased to 2 cc per day equivalent to 20 g of the original solubilized liver. This too proved to be inadequate and within two weeks the monkeys became cachetic and developed anorexia. Monkeys 217 and 219 were

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Hutchings of Lederle Laboratories, Inc., Pearl River, N.Y., for the synthetic *Lactobacillus casei* factor; to Mrs. Edith Jones for assistance in the differential leucocyte counts; to Mr. T. D. Luckey for the B_{10} and B_{11} assays; and to Mrs. Lillian Alberty for the folic acid assays.

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given 2 cc of the crude norite eluate concentrate daily equivalent to 10 g of the original solubilized liver in place of the dialysate, and within a week they began to gain weight rapidly, and the cachexia soon disappeared. Four weeks later the norite eluate concentrate level was reduced to 1 cc per day equivalent to 5 g solubilized liver, and the monkeys continued to grow at the same rate.

Monkey 218 developed a severe diarrhea and refused to eat the ration. Since folic acid-deficient monkeys occasionally develop dysentery, this monkey was given 1 g of sulfasuxidine per day. In addition the M-2 basal was supplemented with 3% whole liver powder which was fed together with the sulfasuxidine by stomach tube. After a week the diarrhea disappeared, and the monkey regained its appetite so that the tube feeding was no longer necessary. Shortly thereafter a sharp increase in weight occurred, and the monkey continued to gain at a rapid rate. Apparently a concentrate showing high vitamin B_{10} and B_{11} activity is inadequate as a source of the essential factor for the monkey. On the other hand the crude norite eluate concentrate carries the active factor since it caused a resumption of growth when fed to the deficient monkeys.

Studies with Crystalline B_c, B_c *Conjugate, and Synthetic* Lactobacillus casei *Factor.* The same monkeys (No. 217, 218, and 219) were used in this study and were returned to the basal ration after having been on the folic acid complete ration for 3 months. In the case of monkeys 217 and 219 the norite eluate concentrate was discontinued while the whole liver powder was removed from the diet of monkey 218. After 10 weeks on the basal ration the growth rates of these 3 monkeys decreased considerably. At this point monkey 217 had a white blood cell count of 8,000 per cmm; monkey 218 5,000 per cmm; and monkey 219 5,000 per cmm. These low counts and the reduced growth rates were good indications that a characteristic "folic acid" deficiency existed.[1] A week later the animals began to lose weight, and accordingly therapy with B_c and B_c conjugate was instituted. 100 γ of vitamin B_c per day was added to the ration of monkey 217, and 200 γ B_c conjugate to the ration of monkeys 218 and 219. White blood cell counts were made 3 times a week.

Preliminary results showed that the free vitamin B_c caused a more rapid response than an equivalent amount of the conjugate. However, it soon became apparent that a complicated deficiency existed in two of the monkeys and so individual case histories are given for purposes of clarification.

No. 219. After 2 weeks of supplementation with 200 γ B_c conjugate the white blood cell count rose from 5,000 to 11,000 per cmm and the animal maintained its weight. During the next week the monkey showed a small increase in weight. The B_c conjugate level was then increased to 300 γ per day, but this change had no additional effect either on the white blood cell level or weight. Two weeks later the monkey started to lose weight but the white blood cell count did not fall. 100 γ B_c per day was substituted for the B_c conjugate but the monkey continued to lose weight during the next 2 weeks. Substituting 150 γ of the synthetic *Lactobacillus casei* factor for the B_c for one week also proved ineffective in checking the decline in weight. Hemoglobin determinations showed that the monkey was anemic, the values averaging 8 g of hemoglobin per 100 cc of blood. Differential white cell counts indicated a definite reversal in the neutrophile-lymphocyte ratio. These changes are typical symptoms of a deficiency of the monkey anti-anemia factor.[11,12] Since the leucocyte count was 16,000 per cmm it seemed unlikely that a concomitant folic acid deficiency existed. Whole liver powder, a good source of the monkey anti-anemia factor,[13] was then fed at a level of 3% of the ration. The monkey rapidly increased in weight, gaining 600 g in one week, and the hemoglobin level rose to 12.2 g per hundred cc. The neutrophile-lymphocyte ratio returned to normal within 2 weeks. Evidently the B_c conjugate, B_c, and the synthetic *Lactobacillus casei* factor were effective in combatting the

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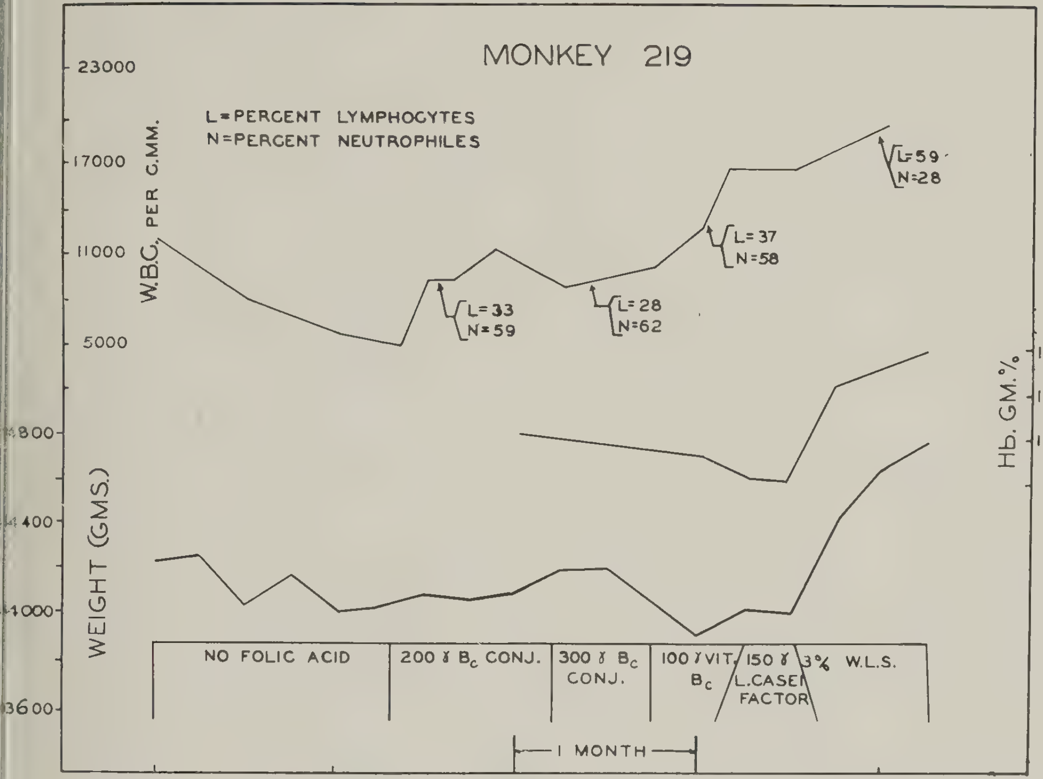

Fig. 1.

The effect of B_c conjugate, vitamin B_c, the synthetic *L. casei* factor and whole liver powder on the growth, leucocytes, and hemoglobin of a monkey with a complicated folic acid deficiency.

The levels of the various supplements were fed daily for the period of time indicated on the graph.

γ B_c conj. = μg of B_c conjugate.

γ Vit. B_c = μg of vitamin B_c.

γ *L. casei* factor = μg of synthetic *Lactobacillus casei* factor.

W.L.S. = whole liver substance (whole liver powder).

folic acid deficiency but whole liver powder was necessary in addition for the monkey to recover from the monkey anti-anemia factor deficiency which was precipitated. The data are summarized in Fig. 1.

No. 218. The ration of this animal was supplemented with 200 γ B_c conjugate. During the next 10 days the monkey continued to lose weight, and the leucocyte count did not rise significantly. Hemoglobin determinations showed that the animal was not anemic since the values were in the range of 14.3 g per hundred cc. The monkey was then given 100 γ B_c per day instead of the conjugate, and within a week the monkey increased in weight and the leucocyte count increased to 18,000 per cmm. The weight gain continued as did the rise in leucocytes, and after 4 weeks the synthetic *Lactobacillus casei* factor was substituted for the B_c at a level of 100 γ per day. The weight increase continued at the same rate and the leucocyte count eventually reached 26,000 per cmm. Differential leucocyte counts showed a normal neutrophile-lymphocyte ratio. In this case there appeared to be no complicating deficiencies. The B_c conjugate at a level of 200γ per day proved to be insufficient in correcting the folic acid deficiency; on the other hand 100 γ B_c per day was quite effective as was the synthetic *Lactobacillus casei* factor. Since this monkey had been given a ration containing 3% whole

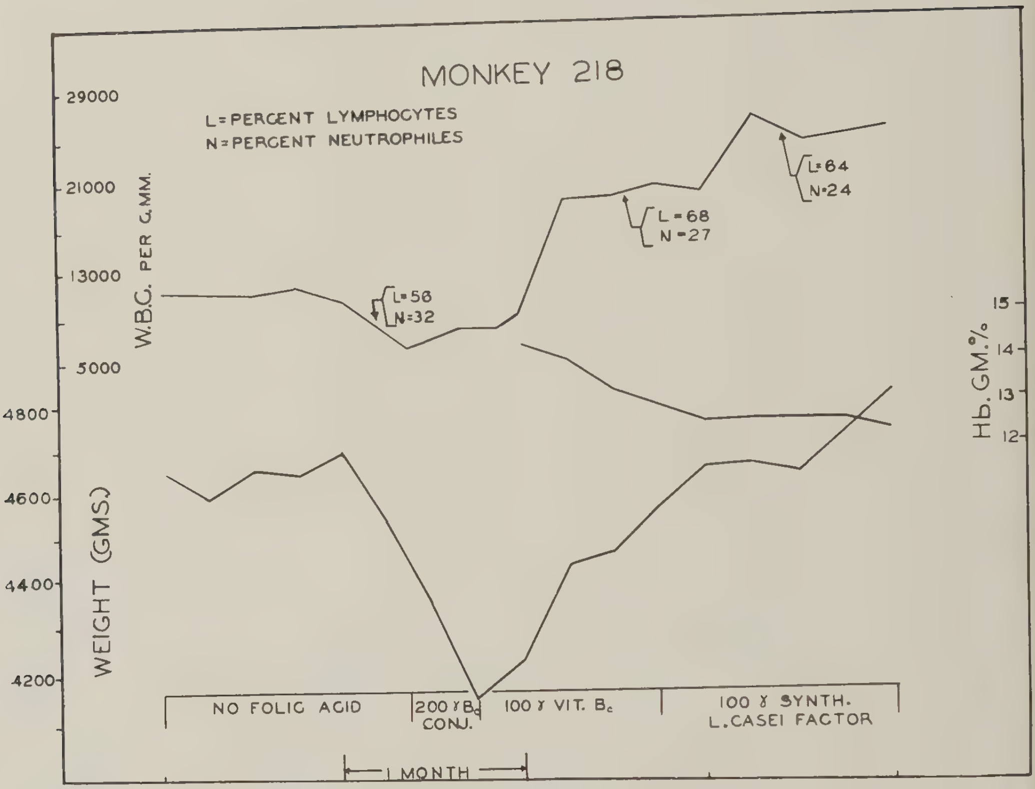

Fig. 2.

The effect of B_c conjugate, vitamin B_c, and the synthetic *L. casei* factor on the growth, leucocytes, and hemoglobin of a monkey with an uncomplicated folic acid deficiency.

The levels of the various supplements were fed daily for the period of time indicateed on the graph.

γ B_c conj. = μg of B_c conjugate.
γ Vit. B_c = μg of vitamin B_c.
γ *L. casei* factor = μg of synthetic *Lactobacillus casei* factor.

liver powder for 3 months before it was placed on the M-2 basal, it is very likely that it had an adequate store of the monkey anti-anemia factor and developed an uncomplicated folic acid deficiency. The data for this animal are summarized in Fig. 2.

No. 217. The weight loss in this monkey was arrested after the animal received 100 γ of B_c per day for 1 week and the leucocyte count rose to 13,000 per cmm. During the next 3 weeks the leucocytes remained at a level of 14,000 per cmm but there was only a slight increase in weight. The level of B_c was increased to 150 γ per day and during the next 10 days the weight increased at a slightly greater rate. At this time the leucocyte count was 20,000 per cmm. 150 γ of the synthetic *Lactobacillus casei* factor per day was then substituted for the B_c and the monkey continued to grow at the same rate and maintained its leucocyte count. Hemoglobin values ranged between 11 and 12 g per 100 cc for this monkey and differential counts showed a reversed neutrophile-lymphocyte ratio. These together with the poor growth rate indicated the existence of a complicating deficiency of the monkey anti-anemia factor. The leucocyte count on the other hand responded to the B_c and *Lactobacillus casei* factor.

Discussion. The crude norite eluate concentrate has been used in our earlier work as a source of folic acid in our purified monkey ration. Since it is desirable to substitute as soon as possible, crystalline compounds for crude concentrates in nutritional studies, folic acid-like compounds were tested for activity. Vitamin B_{10} and B_{11} concentrates which are prepared from the crude norite eluate apparently do not possess activity for the monkey but do contain compounds which are available for the chick. Crystalline vitamin B_c or the synthetic *Lactobacillus casei* factor can replace the norite eluate concentrate in the ration of the monkey each possessing about equal activity. Evidently the B_c conjugate is less active than either of these two compounds.

A deficiency in the monkey anti-anemia factor complicated the folic acid deficiency; similar conditions have been observed in riboflavin,[11] B_6 and pantothenic acid[42] deficiency in the monkey. This deficiency is characterized by a suboptimal hemoglobin level, lack of growth and a reversal in the neutrophile-lymphocyte ratio. In the normal monkey the lymphocytes constitute 60-70% of the total leucocytes and the neutrophiles 25-35%. However, in monkeys deficient in the monkey anti-anemia factor the ratio is reversed and the neutrophiles rise to 60-70% of the total leucocytes and the lymphocytes fall to 25-35% while the total leucocyte count is not appreciably reduced. There is, therefore, an actual increase in the number of neutrophiles and a decrease in the number of lymphocytes. Therapy with whole liver corrects this reversal, and produces a normal hemoglobin level and a resumption of growth.

The folic acid deficiency evidently precipitated a deficiency of this factor in the monkeys which had never received a ration containing whole liver. Thus in monkey 219 the low leucocyte count due to folic acid deficiency was corrected by vitamin B_c but whole liver was necessary to correct the neutrophile-lymphocyte ratio reversal, the low hemoglobin levels and the loss in weight. On the other hand monkey 218 was fed 3% whole liver powder in the ration for 3 months and consequently had an adequate store of this factor when the folic acid deficiency developed. This animal showed the best response to the various crystalline supplements since the folic acid deficiency was uncomplicated. Monkey 217, like monkey 219, showed a good leucocyte response to therapeutic doses of vitamin B_c. The weight response on the other hand was poor and blood studies showed that a dyscrasia existed typical of a deficiency of the monkey anti-anemia factor.

Summary. Vitamin B_{10} and B_{11} concentrates were inactive as a source of folic acid for the monkey.

Crystalline vitamin B_c and the synthetic *Lactobacillus casei* factor were active as a source of folic acid for the monkey when fed at a level of 100 γ per day. The B_c conjugate had less activity when fed at levels of 200 and 300 γ per day.

Folic acid deficiency precipitates a deficiency for the monkey anti-anemia factor which is characterized by lack of growth, suboptimal hemoglobin levels and a reversal in the lymphocyte-neutrophile ratio.

15237

A Differential, Microbiological Assay for O-Heterobiotin.*

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O-Heterobiotin (an analog of biotin in which the sulfur atom has been replaced by an oxygen atom) has biotin-like activity for *L. casei*,[1,2,3] *S. cerevisiæ*,[1,2,3] *R. trifolii*,[4] *L. arabinosus*,[3,5] chicks,[3] and for counteracting egg white toxicity in rats.[3,5] Hofmann *et al.* have developed an assay for O-heterobiotin with *S. cerevisiæ* after the biotin in the sample

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has been destroyed by permanganate oxidation.[4] In an attempt to develop a differential microbiological assay for O-heterobiotin we have tested its activity for representative microörganisms from several different genera. The results with *L. arabinosus* 17-5 and *S. faecalis* R are presented here.

Experimental. The general procedures used and the preparation of the basal medium constituents are similar to those used in the assay of folic acid.[6] A 24-hour-old inoculum of each microörganism is centrifuged and resuspended separately in sterile saline solution. One drop of each suspension is inoculated separately into duplicate sterilized tubes which contain 5 ml of basal medium (Table I), the sample, and distilled water to give a total volume of 10 ml. Standard curves with biotin and O-heterobiotin are run simultaneously with graded levels of the sample. The tubes are incubated at 30° for 16-20 hours and the growth is measured with the Evelyn photoelectric colorimeter.

Since the activity of O-heterobiotin for *L. arabinosus* may vary from one assay to another, a factor relating the activity of O-heterobiotin to that of biotin must be obtained for each assay. An average of 3-5 ratios obtained from different sections of the standard curves gives a reliable activity factor since the ratios are constant within each assay.

TABLE I.
Medium for O-Heterobiotin Determination.

Constituent	**Amt for 10 tubes** (100 cc of complete medium or 50 cc basal)	
Casein	0.5	g
Tryptophane	0.02	
Cystine	0.01	
Glucose	1.0	
Na acetate	0.4	
K_2HPO_4	0.5	
Speakman's salts B	0.5	cc
Adenine • SO_4	1.0	mg
Guanine • HCl	1.0	
Xanthine	1.0	
Uracil	1.0	
Thiamin • HCl	50	γ
Riboflavin	50	
Nicotinic acid	80	
Pyridoxine • HCl	50	
Pyridoxal • HCl	0.2	
α-Pyracin*	0.2	
Biotin	00.0	
Synthetic folic acid†	0.2	
Ca pantothenate	50	
p-Aminobenzoic acid	10	
Water	50	cc
pH	6.8	

* 5-Pyridoxic acid, or the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine are other names for this compound obtained from Merck and Co. through the courtesy of Dr. Karl Folkers.

† Obtained through the courtesy of Dr. B. L. Hutchings at Lederle Laboratories, Inc.

The biotin content of a sample may be determined directly from the standard biotin curve obtained with *S. faecalis*. The O-heterobiotin content may be calculated by multiplying the activity factor for O-heterobiotin by the difference between the apparent biotin content as measured with *L. arabinosus* and the biotin content obtained with *S. faecalis*. These calculations may be summarized:

O = k (Ba-Bt), where
O = O-heterobiotin content of the sample,
k = activity factor for O-heterobiotin compared to biotin with *L. arabinosus*, or

$$= \frac{\text{amount of O-heterobiotin needed to produce x growth}}{\text{amount of biotin needed to produce x growth}}$$

Ba = apparent biotin content as determined with *L. arabinosus*, and
Bt = true biotin content obtained with *S. faecalis*.

The following procedure is used to liberate bound biotin: Add one gram of sample to 25 ml of $2N/H_2SO_4$, autoclave at 122° for 2 hours, neutralize with $4N/NaK(OH)_2$ (a mixture of sodium and potassium hydroxide is used to keep a balance of these ions in the medium), filter and dilute as needed. When a

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for the crystalline vitamins and Dr. S. H. Rubin and Dr. J. A. Aeschlimann of Hoffmann-LaRoche, Inc., Nutley, N.J., for the O-heterobiotin.

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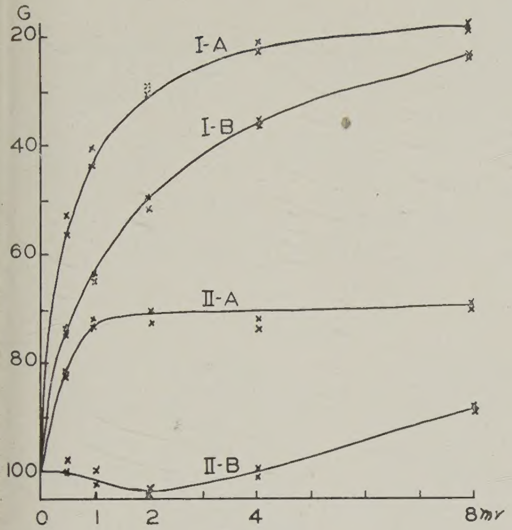

FIG. 1.

Standard curves with biotin (A) and O-heterobiotin (B) obtained with *L. arabinosus* (I) and *S. faecalis* (II).

* G = Galvanometer reading with inoculated blank set at 100.

TABLE II.
Effect of pH on the Activity of *d,l*-O-Heterobiotin.

pH	% activity compared to *d*-biotin *S.f.**	 *L.a.*†	Tubes used for each value
6.6	0.0	33	20
6.8	1.7	44	40
7.0	2.5	39	40
7.3	7.5	29	20

* *S.f.* = *Streptococcus faecalis.*
† *L.a.* = *Lactobacillus arabinosus.*

small amount of O-heterobiotin was hydrolyzed its activity for *L. arabinosus* decreased slightly.

Throughout this work *d*-biotin has been compared to *d,l*-O-heterobiotin. If only the *d*-form of O-heterobiotin is active, the values given here should be increased correspondingly.

Results. A typical set of curves for the assay of O-heterobiotin (Fig. 1) indicates that its activity relative to biotin is constant throughout the curves obtained with *L. arabinosus*. Low levels of O-heterobiotin are somewhat inhibitory for *S. faecalis* while higher levels are about 2% active. The activity of O-heterobiotin for *S. faecalis* may be disregarded since any amount of O-heterobiotin which will produce appreciable growth for this organism (more than 8mγ) gives maximum growth for *L. arabinosus.*

In an attempt to find why the activity factor for O-heterobiotin varied with different assays, standard curves were run at various pH levels. The results (Table II) indicate that the activity of O-heterobiotin relative to biotin for *S. faecalis* increases as the pH is increased from 6.6 to 7.3, while the activity of O-heterobiotin relative to biotin for *L. arabinosus* is greatest at pH 6.8. Thus, pH 6.8, which was originally used, gives the greatest differentiation.

In order to test the effect of biotin upon the activity of O-heterobiotin graded levels of O-heterobiotin were added to tubes containing a known amount of biotin. The amount of O-heterobiotin found corresponded well with the amount added (Table III). The amount of biotin found in tubes containing large amounts of O-heterobiotin was greater than the amount of biotin added. This may be due to an increased sensitivity in both bacteria for O-heterobiotin in the presence of biotin.

When certain natural materials were analyzed for O-heterobiotin the results (Table IV) indicated that if this compound occurs naturally its extent is limited. When O-heterobiotin was added to fresh kidney, after acid hydrolysis, the biotin content as measured with *S. faecalis* was identical to the value obtained for kidney without the added O-heterobiotin. The apparent biotin content, as measured with *L. arabinosus* was higher. When the values obtained were applied in the above formula, the recovery of O-heterobiotin was very satisfactory (Table IV, No. 8).

Discussion. The name O-heterobiotin is used in this paper in conformity with the first common name to appear in the literature[1] rather than the later name oxybiotin[2] since the latter does not definitely distinguish between this compound and such compounds as biotin sulfoxide and biotin sulfone.

Since O-heterobiotin does not seem to occur naturally, biotin values previously obtained are probably not invalidated by this new compound. However, there does seem to exist naturally a compound in chicken muscle and fresh lamb kidney which has more biotin

TABLE III.
Recovery of O-Heterobiotin.

Biotin per tube, mγ	O-heterobiotin per tube, mγ	Biotin found,* mγ	O-heterobiotin found,† mγ	% recovery of O-heterobiotin
0.5	0.5	0.5	0.48	96
0.5	1	0.6	1.14	114
0.5	1	0.6	0.96	96
0.5	2	0.75	2.10	105
0.5	4	0.75	4.35	109

* *S. faecalis* value.
† (*L.a.* value—*S.f.* value) × factor = O-heterobiotin.

TABLE IV.
O-Heterobiotin Content of Naturally Occurring Materials.

Material	γ "Biotin"/g		γ O-heterobiotin/g
	S. faecalis	*L. arabinosus*	
1. Fresh beef liver	1.2	1.1	0.0
2. Fresh chick liver	0.90	0.88	0.0
3. Fresh chick leg muscle	0.15	0.02	0.0
4. Difco yeast extract	3.3	3.3	0.0
5. Grass juice powder	0.40	0.39	0.0
6. Vitab	1.0	0.93	0.0
7. Fresh lamb kidney	0.90	0.66	0.0
8. Fresh lamb kidney + 1 γ O-heterobiotin	0.90	1.61	1.0

activity for *S. faecalis* than for *L. arabinosus*. When O-heterobiotin is fed or injected into animals, it might be expected to occur to some extent in the tissues.

Summary. A differential microbiological assay for O-heterobiotin with *S. faecalis* R and *L. arabinosus* 17-5 has been developed. Assays of several natural materials indicated that O-heterobiotin does not occur generally, but that a metabolite does exist in animal tissues which has biotin activity for *S. faecalis* and not for *L. arabinosus*.